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Pathogenesis of Cell Injury by *Rickettsia conorii*

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This work was undertaken to determine the pathogenic mechanism by which Rickettsia conorii causes disease. R. conorii, an organism that has been neglected in spite of its widespread distribution and pathogenic qualities, was studied in human subjects, animal models, and in vitro. The purpose of the work is to elucidate the pathology of boutonneuse fever and the pathogenic mechanisms which might be blocked therapeutically or prophylactically by examination for presence and severity of specific pathologic features. The study yielded the following: cutaneous necrosis was present in 10 of 13 evaluatable taches noires; vasculitis ~~was severe or moderate in all 14~~; thrombosis was severe in only 1, moderate in only 1, mild in 4, and absent in 10; dermal edema was moderate in 12, and mild in 4. The predominant leukocytes were lymphocytes and macrophages; immunofluorescent Rickettsia conorii were demonstrated in 12.

These results indicate that vascular injury by rickettsiae is the major lesion and that dermal edema is the important result. Thrombosis was generally absent or only focal and mild.

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Summary

This work was undertaken to determine the pathogenic mechanism by which Rickettsia conorii causes disease. R. conorii, an organism that has been neglected in spite of its widespread distribution and pathogenic qualities, was studied in human subjects, animal models, and in vitro. The purpose of the work is to elucidate the pathology of boutonneuse fever and the pathogenic mechanisms which might be blocked therapeutically or prophylactically by examination for presence and severity of specific pathologic features. The study yielded the following: cutaneous necrosis was present in 10 of 15 evaluatable taches noires; vasculitis was severe or moderate in all 16; thrombosis was severe in only 1, moderate in only 1, mild in 4, and absent in 10; dermal edema was moderate in 12, and mild in 4. The predominant leukocytes were lymphocytes and macrophages; immunofluorescent Rickettsia conorii were demonstrated in 12.

These results indicate that vascular injury by rickettsiae is the major lesion and that dermal edema is the important result. Thrombosis was generally absent or only focal and mild.

Seven consecutive Sicilian patients with boutonneuse fever who consented to liver biopsy had hepatic lesions. This suggests that R. conorii is frequently viscerotropic and in patients with particular risk factors poses a serious threat. Clinicoepidemiologic studies with European collaborators depict boutonneuse fever as geographically widely distributed and at times quite severe. We may conclude that the pathogenic mechanisms and pathophysiology of R. conorii infection have been defined at the tissue level and should be studied at the cellular level.

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Foreword

For the protection of human subjects, the investigator has adhered to policies of applicable Federal Law 45CFR46.

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978.

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Statement of Problem

Spotted fever group rickettsiae including Rickettsia conorii, R. sibirica, and R. akari are important potential causes of military health problems. In order to meet the challenges of these diseases to the health of groups of soldiers who enter zoonotic areas, methods of effective prevention, improved diagnosis, and optimal treatment are required. Development of an effective vaccine offers the best hope for prevention of boutonneuse fever and other spotted fever group rickettsioses. No effective vaccine exists for any of these rickettsial diseases. Because most effective vaccines for prokaryotic organisms rely upon interdiction of the specific pathogenic mechanism of the organism, e.g., diphtheria and tetanus, it is important to elucidate the pathogenic mechanism of cell injury by R. conorii. The failure of killed rickettsial and bacterial vaccines, e.g., Rocky Mountain spotted fever, typhoid fever, and cholera, may be a result of a lack of stimulation of the immune system to block crucial pathogenic steps. The goal of this research contract is to determine the pathogenic mechanism for R. conorii. Laboratory research on hypothetical rickettsial pathogenic effects must be compared with observations on the human disease in order to assure as well as possible the relevance and reality of working models of the host-parasite interaction. The problems of lack of information on the pathology of boutonneuse fever, the pathogenic mechanisms of R. conorii, and the composition of the immune and inflammatory cell populations actually present in foci of rickettsial infection in humans are addressed in this research project. Diagnosis of boutonneuse fever, North Asian tick typhus, and rickettsialpox is an unsure affair with considerable room for error. Misdiagnosis and delayed diagnosis result in prolonged illness, need for more care often including nursing and hospitalization and failure to institute epidemiologic preventive measures. Yet, clinical features are variable and do not always lead to a timely correct diagnosis. There has been no rapid, acute laboratory diagnostic method. Serologic diagnosis is a retrospective tool employed during convalescence or in the late stage of the illness. There are few facilities in the world for isolation of R. conorii, and the laboratory procedure for isolation is both cumbersome and long. A diagnostic test that can be applied during the acute stage of illness is an expected spinoff of this research project.

Background

Rickettsial diseases occur over a wide geographic distribution, are firmly entrenched ecologically, and pose an important threat to both military and public health.

Members of the genus Rickettsia are classified into three groups on the basis of shared group antigens: spotted fever group, typhus group, and scrub typhus group. All are obligate intracellular bacteria which spend at least a portion of their life in arthropod hosts such as ticks, mites, fleas, or lice. They all affect man in a similar fashion with hematogenous spread and infection of vascular endothelium producing increased vascular permeability and vasculitis in multiple organ systems. These rickettsiae include the etiologic agents of diseases that have been documented as major military health problems. Rickettsia prowazekii has affected the outcome of numerous military campaigns for centuries. R. tsutsugamushi was a severe problem in Asia and the western Pacific theaters during World War II and infected soldiers in the Viet Nam War. These rickettsiae have continued to attract research support. Although R. conorii has received far less attention, it too has been docu-

mented as an important cause of illness among troops in South Africa. R. conorii is a member of the spotted fever group of rickettsiae along with other human pathogens including R. rickettsii (Rocky Mountain spotted fever), R. akari (rickettsialpox), R. sibirica (North Asian tick typhus), and R. australis (Queensland tick typhus). Isolates of spotted fever group rickettsiae from the Mediterranean basin, where the disease is known as boutonneuse fever, East Africa (Kenya tick typhus), South Africa (South African tick typhus), and the Indian subcontinent (Indian tick typhus), were all shown to be members of the same species, R. conorii, by the mouse toxin neutralization test and by microimmunofluorescence test. Data presented by Myers and Wisseman on DNA hybridizations among the spotted fever group rickettsiae have documented close relationships among various strains of R. conorii including rickettsiae associated with the severe disease occurring in Israel and R. rickettsii. Many of these hybridizations were in the range of 90-100% homology.

Infection of man with various strains of R. conorii occurs in a widespread geographic distribution in the Old World with well-documented disease in the Mediterranean basin, Africa, and the Middle East from Israel to India. In the Mediterranean basin, the disease is endemic in Portugal, Spain, southern France, Italy, Greece, Romania, Turkey, Morocco, Algeria, Tunisia, Libya, and Egypt as well as in the margins of the Black Sea and the Caspian basin. More recently it has been reported from South Africa, Kenya, the Central African Republic, India, Pakistan, Togo, Ethiopia, Cameroun, and Israel.

In the majority of the areas where the disease is endemic, it is recognized as sporadic cases during the summer months with little variation in the annual numbers of cases reported. Scafidi notes that there were 107 cases in Israel in 1974, around 30 annual cases in Tunisia from 1961-1975, and 20 annual cases in Marseille from 1925-1930. He and Bourgeade et al, however, point out that these numbers do not reflect the reality since the great majority of patients are treated at home and are not reported. This is also one explanation for the scarcity of information about the prevalence of the disease.

The low endemicity that prevails in the majority of the affected areas has changed significantly in Italy where, since 1975, there was a sharp increase in the incidence of the disease. Indeed, from an average of less than 10 cases per year up to 1972 the number of cases in Sicily increased progressively to reach 219 cases in 1979. Similar increases were observed in other regions of Italy as Liguria, Sardinia, and Lazzio; in this last mentioned region that includes the city of Rome, there were 369 cases reported in 1979 . Besides in Rome, the disease has also been reported in suburban and urban Marseille, and there are data that it is also increasing in Spain and Portugal. A large number of reports of boutonneuse fever have been published recently in Spain. Many cases are seen in southern France around Marseille every year.

The causes for such a rapid increase in the incidence of boutonneuse fever in Italy are not apparent. The Italians have suggested several possible explanations: 1) increase in the vector tick population, 2) introduction of new vectors, and 3) changes in the ecosystem. There have been some very interesting observations on the isle of Ustica where, after the recent introduction of wild rabbits, there was an explosive proliferation of Hyalomma excavatum, a tick that had rarely been found in the island previously. Gilot, et al also mention the possibility of adaptation of certain species of ticks, parasites of wild animals, to human dwellings and the potential consequences of the transmission of boutonneuse fever.

What is happening in Italy, France and Spain may occur in other regions. Weyer, reviewing the subject of rickettsioses in 1978, said, "Despite the great successes in control, none of the rickettsioses pathogenic for man have been eradicated. Therefore, it is necessary to preserve the knowledge about these once devastating and important diseases because the present situation could change suddenly."

Indeed, recent data have demonstrated that several different species of ticks harbor spotted fever group rickettsiae not only in the known endemic areas but also in regions where the human disease is not recognized including Pakistan, Armenia, Thailand, areas of France, Czechoslovakia, Austria, and Germany.

Boutonneuse fever is transmitted to man from ticks, most frequently by Rhipicephalus sanguineus. Infected ticks transmit the disease through their infected salivary secretions during the bite; exceptionally the agents may invade the human host from infectious tick material through abrasions in the skin or through the conjunctivae. There are references that report the disease being acquired by persons who rubbed their eyes after deticking dogs and, in fact, the principal investigator has observed just such a case. The agent seems innocuous to the tick which also serves as reservoir for R. conorii which is transmitted transovarially in ticks. Small wild mammals are the source of blood meals for immature forms of R. sanguineus. Dogs, and on occasion man, are the source of blood meals for the adult stage. The following species of ticks, besides the common vector Rhipicephalus sanguineus have been reported to harbor R. conorii: Ixodes ricinus, R. hexagonus, Dermacentor marginatus, and D. reticulatus in France; Haemaphysalis leachii, Amblyomma hebraeum, Rhipicephalus appendiculatus, R. evertsi, and Hyalomma marginatus rufipedes in South Africa; Amblyomma variegatum and Hyalomma albiparamatum in Kenya; Ixodes granulatus in Malaysia; Rhipicephalus simus, Amblyomma variegatum, A. cohaerens, and A. gemma in Ethiopia; and Rhipicephalus bursa, Hyalomma marginatum, H. lusitanicum, and Haemaphysalis punctata in Sicily. Moreover, serological tests in wild and domestic animals have shown that antibodies against R. conorii are present in several species in many regions, some of them far away from the known endemic areas. In Sicily, 20% of dogs harbor R. sanguineus and 29-71% of them have antibodies to R. conorii identified by indirect immunofluorescence assay. Serologic tests have identified antibodies against R. conorii in large numbers of healthy persons: in Africa, 13% of sera contained antibodies in an investigation in Cameroun and similar results were reported from Niger, Zaire and Central African Republic; in Greece 16% of 560 sera from healty persons were positive; data from France indicate that positive serology in healthy persons has been observed in Caen, Nantes, and Lyon. In one endemic area of Sicily 19.3% of healthy subjects had positive immunofluorescence assay for anti-R. conorii antibodies. Not all of these studies employed the same serological tests, and there is variation in specificity among different tests. Some, however, used specific immunofluorescence techniques.

All the data above presented confirm the suggestion of Weyer that the stage is set for an increase in the frequency of boutonneuse fever and that this may occur in many different areas of the world.

Recently there have been reports of cases of boutonneuse fever in German and Swiss tourists who had returned from endemic areas and even of cases in American tourists returning from Africa. Interestingly, a tick was found on one of these patients that might, if circumstances had been favorable, have become established in an American ecological niche. Cases have also been reported in persons living in Paris and other parts of Europe that are not near the Mediterranean Sea at times in foci associated with R. sanguineus-

infested dogs and houses resulting from travel with pets to endemic areas.

Human illness caused by R. conorii infection is usually an incapacitating febrile exanthem. Death has been reported more frequently in recent years, and some strains of R. conorii possess the capability of producing severe disease requiring hospitalization and critical medical and nursing care. The disease usually resolves spontaneously in one or two weeks, this period being reduced by appropriate antibiotic therapy which may be given at home. It is necessary to emphasize that even when mild the illness is incapacitating and in a minority of cases can be severe or even fatal; moreover, in certain regions, as apparently is the case in Israel, South Africa, France, and Spain, it can assume a more severe course similar to the picture of Rocky Mountain spotted fever. Severe disease has been associated with G6PD deficiency, alcoholism, older age, and diabetes. Men are slightly more frequently affected than females, and the disease occurs at all ages being, however, uncommon in the very young and very old. Most of the patients report contact with dogs, ticks, or recent visit to endemic areas; others are farmers or hunters. The incubation period varies from 7 to 14 days, but can be as short as 4 or as long as 22 days. In the majority of the cases the patient remembers being bitten by a tick and from 33% to 92% of them have an eschar (tache noire) at the site of the tick bite. Less frequently they have acute unilateral conjunctivitis.

The disease begins with sudden increase in temperature to levels as high as 40°C; at the same time the patients complain of joint and muscle pain and violent, persistent headache that is frequently retroorbital. There is also congestion of the conjunctivae and mild lymphadenopathy. These manifestations coincide with the appearance of the eschar. Four to five days after the beginning of the fever the typical rash appears; it is first observed on the limbs but rapidly expands to trunk and face with palms and soles also being involved. In some cases even the oral mucosa presents an exanthem. In the beginning the rash appears as erythematous macules that rapidly change to a maculopapular pattern and eventually become nodular or button-like, as the name describes. The early lesions are light pink, but some of the older ones may become darker or hemorrhagic. The rash occurs in successive bouts so that lesions in different phases may be observed side by side.

Fever persists for 7-14 days, and during this period 46% of the patients develop splenomegaly, 20% hepatomegaly, and some patients, signs of pulmonary congestion. Diarrhea, constipation and vomiting may also occur. Neurological signs of meningeal irritation as nuchal rigidity or Kernig's sign as well as obtundation and even coma can be observed in a minority of the cases. These more severe manifestations occur mainly in older or debilitated persons; they are exceptional in children. Recovery is usually uneventful without sequelae. Mortality has been, until recently, thought to be quite low. In a few cases, however, complications occur; they are rare and, as stated, tend to occur in older debilitated persons. Scafidi et al describe cases of hypertoxic, "dermatotifosa" and hemorrhagic disease, the last form being associated with severe gastrointestinal or genital bleeding. Fatal gastrointestinal hemorrhage with rickettsial vasculitis of the stomach has been described. Scafidi et al describe cases with atrial fibrillation, myocardial ischemia, and renal complications. A series of French publications describe "atypical rickettsiosis" with pericarditis, pleuritis, and pneumonitis. Some of the cases, however, did not present with eschars and the final diagnosis was made by positive microagglutination tests according to the method of Giroud, thus raising doubts concerning the diagnosis. In Israel, however, there have been some very interesting cases of tick-borne rickettsiosis with severe renal insufficiency requiring dialysis; in these cases, there are questions about

the exact classification of the etiologic agent that did not conform exactly with the antigenic structure of R. conorii. More recently severe and fatal cases have been described in South Africa, Spain, and France.

The clinical feature that is most significant diagnostically in R. conorii infection is the tache noire which develops at the site of tickbite in approximately 50% of cases. The tache noire, or black spot, is a zone of dermal and epidermal necrosis which may appear prior to onset of fever and rash. Conor and Bruch did not describe eschars in the original report of human R. conorii infection in 1910. Tache noire is a French term and was introduced in 1925 by Pieri to refer to the tickbite site eschar in boutonneuse fever. Thereafter, the term tache noire seems to have been used continuously. Similar eschars are frequently observed in scrub typhus (R. tsutsugamushi), North Asian tick typhus (R. sibirica), rickettsialpox, (R. akari), and Queensland tick typhus (R. australis). Eschars are rarely observed in Rocky Mountain spotted fever and do not occur in typhus fever and murine typhus. Thus, eschars are seen only in rickettsioses transmitted by inoculation of infected salivary secretions by ticks and mites and are not observed in rickettsioses transmitted by scratching rickettsia-containing louse or flea feces into the skin. Patients who develop boutonneuse fever after accidental introduction of infected tick constitutents into the conjunctiva do not have eschars, but manifest conjunctivitis at the portal of entry.

Our laboratory has described the clinical features, brightfield microscopic pathology, and distribution of R. rickettsii in eschars which occurred in two fatal cases of Rocky Mountain spotted fever examined at autopsy. These eschars consisted of a 8 x 10 mm oval region of necrotic epidermis and underlying dermis. The necrotic zone was surrounded by a zone of blood vessels that were injured with extensive thrombosis and intramural and perivascular mononuclear inflammatory cells. Immunohistochemical examination revealed very large quantities of R. rickettsii in the endothelium and vascular wall of these blood vessels.

There is some degree of controversy about the role of constituents of tick salivary secretions such as enzymes associated with tickbite in the pathogenesis of the tache noire. Experimental studies suggest that the dose of inoculum of rickettsiae rather than the tickbite itself is crucial. Inoculation of a large dose of R. rickettsii, a generally nonescharogenic rickettsia, into human skin by syringe and needle produces eschars. Inoculation of R. conorii into the skin of syphilitic subjects as pyrotherapy produced taches noires proportional to the quantity of rickettsiae injected. Even nonescharogenic R. mooseri produces eschars in the skin of guinea pigs injected intradermally by syringe and needle with a large dose of rickettsiae. Not all monkeys inoculated with R. tsutsugamushi develop an eschar at the injection site; some develop only papules which do not undergo epidermal necrosis and ulceration. Rabbits inoculated intracutaneously with a high dose of R. sibirica developed an eschar; rabbits inoculated with 1% of the escharogenic dose developed only cutaneous erythema without necrosis or formation of a dark crust. Thus, the tache noire appears to be an accessible lesion that contains the pathogenic mechanisms of R. conorii and the immune and inflammatory mechanisms of the host that lead to healing.

Hypothetical rickettsial pathogenic mechanisms include both those that are host-mediated and rickettsia-mediated. Host-mediated mechanisms of injury which have been proposed include immunopathology, blood coagulation, and inflammation. Rickettsia-mediated mechanisms might include endotoxin, exotoxin, enzymes that destroy host components, metabolic competition for the host's intracellular substrates, ATP parasitism, and host cell membrane injury

on rickettsial penetration into and/or release from the target cell.

Experimental evidence indicates that host-mediated pathogenic mechanisms such as immunopathology, Shwartzman phenomenon-like blood coagulation, and inflammation are not the primary mechanisms of injury in infection by R. rickettsii. Localized effects of kallikrein are probably events secondary to the primary pathogenic mechanism(s). Occlusive vascular thrombosis is infrequent and has not been demonstrated as a primary pathogenic mechanism.

Among the hypothetical rickettsia-mediated mechanisms of injury, currently no toxin of R. rickettsii has been identified, and there is evidence against the existence of a toxin as an important pathogenic mechanism. The confusion regarding this hypothesis has originated in the so-called mouse toxin phenomenon and in erroneous analogies drawn between endotoxin and rickettsiae. Mouse toxicity depends on viable, metabolically active rickettsiae and is prevented by heating (60°C for 30 minutes), exposure to dilute formalin, rickettsial starvation, ultraviolet irradiation, specific anti-rickettsial antiserum neutralization, and a beta-lipoprotein present in some normal human sera. The pathogenesis of this phenomenon may be related to the pathophysiology of the rickettsia-host cell interaction, e.g., massive rickettsial penetration of endothelium. Rickettsiae of both the typhus and spotted fever groups have been shown to contain lipopolysaccharides. However, the endotoxin activity in bioassays including the Shwartzman phenomenon and Limulus assay was considerably less than that of potent bacterial endotoxins. Moreover, study of the adrenal in fatal RMSF has not demonstrated the pathologic lesions expected of endotoxin-mediated pathogenesis. Further evidence against the hypothesis of rickettsial toxin has been demonstrated in the plaque model. Thus, the evidence for a rickettsial toxin of pathogenic importance is quite meager.

The plaque model has been established as a useful tool for investigation of pathogenic mechanisms of cell injury by R. rickettsii. Inoculation of confluent monolayers of primary chick embryo cells derived from 12-day old specific pathogen-free, antibiotic-free, embryonated hen's eggs with a defined quantity of R. rickettsii results in a predictable course of infection and pathologic alterations in vitro. Each infectious unit under agarose overlay produces contiguous centrifugal spread of intracellular infection and injury to the host cell monolayer. This model produces a grossly visible plaque on day 5 after inoculation when a second overlay of agarose-containing the supravital dye neutral red is added. The plaque provides a temporal and spatial cross-section of the rickettsia-host cell interaction including rickettsial penetration, proliferation and release, and host cell cytopathologic alterations and necrosis. Morphometric analysis of the plaque and surrounding infected and uninfected cells has been performed maintaining the topographic relationships of the cells as a monolayer. The results have shown the association of intensity of infection and cytopathology at the microscopic and ultrastructural levels. There is a statistically highly significant relationship between the intensity of infection as measured by the quantity of intracellular rickettsiae and the presence of cellular injury as judged by cytopathology and necrosis. This relationship is valid independently of the apparent duration of infection. That is to say, more heavily parasitized host cells are more likely to exhibit pathologic alterations, even if they are located at the margin of the plaque, than those cells which contain fewer rickettsiae and are nearer to the center of the plaque. This study also confirms the observation of Silverman and Wisseman that the typical cytopathologic change in chick embryo cells infected with R. rickettsii is distinct dilation of the cisternae of endoplasmic reticulum. This ultrastructural finding is characteristic of the response of an injured cell to the

influx of water. The utilization of the technique of maintaining the topography of the monolayer intact enabled us to determine that the uninfected cells of the monolayer even within 1 mm of the intensely infected marginal zone of the plaque were normal by ultrastructural and supravital dye staining criteria even though they were exposed to the same milieu of extracellular nutritional factors, nonspecific toxic products of metabolism and substances released from injured cells, and senescence of cultured cells. Thus, the plaque model, which has a 0.5% agarose overlay that prevents rapid, distant spread of rickettsiae and yet allows for diffusion of macromolecules, demonstrated that cell injury was limited to the more heavily parasitized cells and that there was no toxic effect on uninfected cells, even those immediately adjacent. This is strong evidence that R. rickettsii does not elaborate an extracellular toxin which affects chick embryo cells. Further studies in our laboratory have extended this observation and conclusion to Vero cells which are of primate origin and to human umbilical vein endothelial cells.

Another strong indication that R. rickettsii does not produce an important toxin resulted from observations utilizing parabiotic chambers. Specially designed flasks contained coverslips with monolayers of cells with fluid overlay in separate chambers which were separated by an 0.22 um millipore filter. R. rickettsii was inoculated into one chamber of several flasks; other control flasks were observed without rickettsiae in either chamber. Inoculated monolayers developed cytopathic effect associated with heavy rickettsial infection. On the other hand, the cells in the opposite chamber remained viable with the same appearance as monolayers of unmanipulated parabiotic chambers. No toxic macromolecules injured the side of the chamber which was protected from rickettsial infection by the 0.22 um filter. The filter offered no barrier to the free passage of molecules between the infected and uninfected chambers. Thus, in an experimental system in which rickettsiae injured infected host cells, we demonstrated no effect of putative toxin, which would have been in equal concentration in the extracellular fluid of both chambers if it were present.

Examination of the hypothesis of competition for metabolic substrates has also failed to produce evidence to support it as a pathogenic mechanism in plaque model experiments with supplemental glutamate and glutamine. Although rickettsiae are capable of generating ATP for penetration of host cells by oxidation of glutamate, exogenous ATP from the host cell is utilized for biosynthesis of proteins and lipids by rickettsiae. This energy parasitism is mediated by an efficient rickettsial ATP/ADP transport system. No experiment has yet been designed and executed to test the hypothesis of energy parasitism as a pathogenic mechanism.

Experiments reported principally by Winkler and co-workers suggest that rickettsial penetration-associated phospholipase activity injures the host cell membrane. The work of Winkler and associates on hemolysis by viable R. prowazekii has led to an understanding of the rickettsia-host cell membrane interaction which probably forms the basis of penetration and a mechanism of cell injury. Rickettsial hemolysis may be divided into two steps, adsorption and lysis. Hemolysis is inhibited by cyanide (1 mM KCN, an inhibitor of the electron transport system), low temperature (0°C), and starvation of R. prowazekii for glutamate. Ghosts of erythrocytes exposed to Amphotericin B or digitonin, compounds which bind to the cholesterol-containing receptor sites in the erythrocytic membrane, are no longer able to adsorb rickettsiae. Adsorption and hemolysis are inhibited by adenine nucleotides, ADP, ATP, arsenite, which is a Krebs cycle inhibitor, and 2,4-dinitrophenol and m-chloro-phenylhydrazone, which are oxidative phosphorylation uncouplers. When rickettsiae are unable to generate ATP by metabolism of glutamate because of

cyanide or arsenite inhibition, added ATP restores hemolytic activity of the rickettsiae. ATP, however, does not restore hemolytic activity inhibited by uncouplers. Fluoride (10 mM NaF) prevents hemolysis by inhibition of erythrocytic glycolysis without affecting adsorption or rickettsial metabolism. Recently, rickettsial hemolysis has been shown to be associated with phospholipase A activity, which resulted in hydrolysis of fatty acids from the glycerophospholipids of the red blood cell membrane. Inhibition of either adsorption or lysis also prevented the release of free fatty acids.

Host cell entry by rickettsiae has many correlates with rickettsial hemolysis. Inactivation of R. tsutsugamushi by heat (56°C for 5 minutes), exposure to ultraviolet irradiation, or incubation with 0.1% formalin prevents penetration into host cells. Penetration of L cells by R. prowazekii comprises two steps, adherence and internalization, and requires active participation by both the rickettsia and the host cell. Treatment of rickettsiae with ultraviolet irradiation, 3% formaldehyde, or 1 mM KCN inhibited adherence to and internalization into L cells. The few inactivated rickettsiae found associated with L cells were mostly adherent rather than internalized. Treatment of L cells with NaF (an inhibitor of metabolism), N-ethylmaleimide, or cytochalasin B inhibited internalization of rickettsiae. Similar studies of the entry of R. prowazekii into endothelial cells support the hypothesis of induced phagocytosis. Inoculation of R. prowazekii onto L cells at large multiplicities of infection induced immediate cytotoxicity. This cytotoxic effect was associated with phospholipase A activity and hydrolysis of fatty acids from host cell phospholipids. Cytotoxicity and phospholipase were inhibited in a parallel manner by KCN, N-ethylmaleimide, NaF, and low temperature.

Further studies in our laboratory of pathogenic mechanisms in the plaque model employed chemical agents, which have a sound theoretical basis of inhibiting rickettsial penetration either at the step of adsorption of the rickettsia to the host cell (Amphotericin B and digitonin) or at the step of internalization associated with phospholipase A activity. These compounds have been demonstrated to reduce plaque formation. Amphotericin B and digitonin have been reported to inhibit the attachment of R. prowazekii to erythrocytic cell membranes by binding to a cholesterol receptor in the membrane. Amphotericin B was introduced in concentrations of 5 and 10 ug/ml to the overlay after the establishment of infected foci on day 4 after inoculation of R. rickettsii. In order to maintain active levels of this drug which has a decay of 50% per 24 hours at 37°C, Amphotericin B was replenished in sequential overlays on days 5 and 6. On day 6 Amphotericin B caused plaque reduction of 42-45% at both concentrations. More plaques appeared on day 7 with plaque reduction of 16-23%. A similar experiment with digitonin at the same concentrations caused similar plaque reduction (38-40%). Plaque reduction was not observed on day 7. When the levels of cholesterol receptor-binding drugs are maintained, plaque reduction can be demonstrated. This suggests that inhibition of rickettsial adsorption delays the cytopathic effect of R. rickettsii in primary chick embryo cells.

Phenterine is a drug which has been shown to have phospholipase A2 inhibitory activity. A dose response study with this drug was performed in the plaque model. Plaque reduction was demonstrated at all doses of phentermine: 69% plaque reduction at 0.5 mg/ml; 54% at 0.1 mg/ml; 25% at 0.05 mg/ml; and 32% at 0.01 mg/ml. These results demonstrate that phentermine reduces the cytopathic effect of R. rickettsii and suggest that phospholipase activity may be a pathogenic mechanism for R. rickettsii. These data extend and support the observations of Winkler that phospholipase activity is associated with hemolysis and immediate cytotoxicity of a large inoculum of R.

prowazekii.

Previous reports have documented that R. conorii forms distinct plaques similar to those of R. rickettsii in the plaque model. McDade et al produced distinct plaques with R. conorii in chick embryo cells with a first overlay of medium 199 containing 5% calf serum and 0.5% agarose and a later second overlay of medium 199, no calf serum, 0.5% agarose, and 0.01% neutral red. Wike et al studied the critical variables in the plaque assay system for rickettsiae and also showed that R. conorii (Malish strain) produced distinct plaques in the standard chick embryo monolayer with nutrient overlay containing agarose. Thus, the plaque model offers an opportunity to examine quantitatively and predictably the pathogenic mechanisms of R. conorii in an in vitro system that may be manipulated experimentally to examine hypotheses such as phospholipase-mediated injury.

Because one hypothetical explanation for the apparent rarity of severe visceral involvement in BF as compared with RMSF (encephalitis, hepatitis, pneumonitis) is lower temperature sensitivity of R. conorii, we are interested in the effects of temperature on the physiology and pathogenicity of the organism. Oaks and Osterman have investigated the effects of temperature on the optimal growth of R. conorii. This species of rickettsia has an optimal range for growth in gamma-irradiated L cells of 32-38°C with inhibited proliferation at 40°C. The low rate of proliferation at 40°C might explain the minimal visceral involvement in febrile patients whose body core temperature is about 38°C and may exceed 40°C. An unanswered question is the effect of temperature on the pathogenic mechanism of R. conorii.

Approach to the Problem

Many features of boutonneuse fever have been investigated to a far less degree than typhus fever and Rocky Mountain spotted fever. In particular, pathogenic mechanisms, immune mechanisms against R. conorii, and the laboratory diagnosis of boutonneuse fever have not been investigated sufficiently. There are advantages of studying human cases, animal models, and cell culture models of R. conorii infection.

The localized lesion at the site of the tick bite, the eschar or tache noire, offers an excellent opportunity to extend our knowledge of pathogenic mechanisms, immune mechanisms, and laboratory diagnosis of BF in humans. In contrast to typhus and Rocky Mountain spotted fever in which the lesions, although numerous and widespread, are extremely focal, the tache noire is sufficiently large and contains a large contiguous network of severely injured blood vessels that was expected to allow predictable sampling and qualitative and quantitative analysis of rickettsial infection, host cell injury, and host inflammatory and immune cellular response. Thus, although the brightfield microscopic lesions are better described in typhus fever, Rocky Mountain spotted fever and scrub typhus than in boutonneuse fever, these reports are not quantitative, often do not demonstrate rickettsiae with the efficiency and specificity of immunohistochemical techniques, and do not evaluate the ultrastructure of the human lesions. Surgically excised, well-fixed eschars allowed this investigational approach in boutonneuse fever.

As yet no significant in vivo ultrastructural study of the human host-rickettsial relationship has been reported. There are two major reasons: 1) the infection in human skin is extremely focal, in the exact center of the maculopapular rash of RMSF and typhus and, thus, is difficult to find by electron microscopy; 2) intensely infected visceral tissues from fatal cases of BF and typhus are not suitable for ultrastructural investigation because of postmortem autolysis that occurs prior to performance of the necropsy.

Surgical biopsy of the tache noire of BF should provide well-preserved lesions that were expected to contain intense R. conorii infection for ultrastructural investigation. A report of the ultrastructural aspects of an eschar in Rocky Mountain spotted fever described rickettsiae in the lesion. However, the published electron micrographs were of poor quality, and no rickettsiae were identifiable in them. Correspondence with the authors directly in an attempt to obtain copies of the original electron micrographs or the EM grids for examination personally has not been answered.

A sample of the tache noire was collected by sterile skin biopsy technique under local anesthesia after obtaining the patient's informed consent. The specimen was divided into three small 1 mm^3 blocks and fixed for electron microscopy by immersion in cold buffered glutaraldehyde-formaldehyde solution. The fixed specimen was held in this solution for the period of shipping from Italy to our laboratory. On arrival at the Infectious Pathogenesis Laboratory in the Department of Pathology of the University of North Carolina, the specimen was postfixed in 1% osmium tetroxide, dehydrated in graded alcohol concentrations, embedded in a mixture of Epon and Araldite, ultrathin sectioned on an ultramicrotome, and stained with uranyl acetate and lead citrate. Sections were examined on a high resolution Zeiss 10 A electron microscope.

The remainder of the specimen was fixed in neutral buffered-4% formaldehyde for routine histology, histochemistry, and immunohistochemistry. Fixed tissue was embedded in paraffin and a ribbon of serial sections was cut at 4 um thickness. Adjacent sections were mounted for staining by hematoxylin- eosin (H & E) for routine evaluation of pathologic lesions, by phosphotungstic acid-hematoxylin (PTAH) for fibrin thrombi, by Voerhoff-van Gieson technique (VV) for evaluation of integrity of vascular elastic tissue, by modified Brown-Hopps (BH) technique for histochemical demonstration of rickettsiae, and by Giemsa technique and methyl green pyronin (MGP) for identification of host immune and inflammatory cells. Among these stains, PTAH and VV yield highly sensitive results, BH demonstrates intracellular rickettsiae but with less sensitivity, consistency, and specificity than immunofluorescence, and Giemsa and MGP assist in identification of eosinophils, basophils, neutrophils, activated lymphocytes, and plasma cells but leave a large portion of unidentified mononuclear lymphocytes.

Adjacent sections from the ribbon were processed for immunofluorescent demonstration of R. conorii. Sections were affixed onto clean glass slides with nonautofluorescent LePage Bond Fast Resin Glue to prevent them from being washed off the slide after digestion with trypsin. Sections affixed to slides with glue were heated in an oven at 60°C for 1 hour, deparaffinized in three changes of xylene for 10 minutes each, and rehydrated through serial changes of ethanol in concentrations of 100%, 95%, 70%, 50%, and 35% and finally in distilled water. Sections were then incubated in 0.1% trypsin with 0.1% $CaCl_2$, pH 7.8, at 37°C for 4 hours. The slides were washed thoroughly in distilled water, washed for 30 minutes in phosphate-buffered saline, and reacted with the specific immunofluorescent system for R. conorii. We used anti-SFG rickettsial conjugate in the direct immunofluorescence system to demonstrate structures which have the expected vascular location and coccobacillary morphology of rickettsiae.

In order to evaluate the cellular immune response to R. conorii in taches noires by specific immunohistochemistry, six unselected, adult patients (3 males and 3 females) with MSF and neither comcomitant diseases nor clinical or serological evidence of previous contact with R. conorii were studied in the University Hospital of Salamanca (Spain) during 1985. Clinical diagnosis (based on the characteristic signs and symptoms and on the presentation of the

cases in endemic areas during the summer months) was confirmed serologically by means of indirect immunofluorescent antibody test against R. conorii (titers over 1/640 in convalescent sera and four-fold increase in the level of antibodies). All of the clinical courses evolved favorably.

With the informed consent of the patients, we obtained biopsy samples of the taches noires at the moment of diagnosis immediately before the beginning of antibiotic therapy. A portion of each specimen was frozen in liquid nitrogen and maintained at -70°C during storage and transport to the University of North Carolina at Chapel Hill. The remaining portions were processed for study by other histological procedures. Four um frozen sections of each sample were placed on microscope slides, air dried for 30 min., fixed in absolute acetone at room temperature for 3 seconds, air dried and stored at -20°C prior to staining.

Immunohistochemical staining of the slides followed fixation in cold acetone at -20°C for 20 min and air drying at room temperature. Slides were incubated in phosphate buffered saline (PBS) for 20 minutes at room temperature, incubated with normal horse serum for 30 min in a moist chamber, excess of serum blotted, and then incubated with the optimal dilution (previously determined through tests in human lymph nodes which had been diagnosed as nonneoplastic hyperplasia) of monoclonal antibody for 30 min in a moist chamber. We used the mouse monoclonal antibodies anti-leu-4, anti-leu-3a, anti-leu-2a, anti-leu-12 and anti-leu-M3 (Becton Dickinson, Mountain View, California) which recognize, respectively, the human cellular populations: all T lymphocytes, T helper/inducer subset, T cytotoxic/suppressor subset, B lymphocytes, and macrophages. Slides were washed twice for 7 min. each with gentle agitation in PBS and then incubated for 30 min. in a moist chamber with FITC-conjugated rabbit antimouse immunoglobulin (Accurate Chemical and Scientific Corp., Westbury, NY) which had been diluted 1/40 and centrifuged for 10 min. at 2000 g to remove aggregates of conjugate. Slides were then washed in PBS. Control frozen sections from lymph nodes were included in each staining procedure. We examined the slides, mounted in a glycerol-PBS solution, with a Leitz Orthoplan ultraviolet microscope with FITC barrier and exciter filters equipped for epi-illumination and photomicrography. Three or 4 major areas of perivascular inflammatory infiltrate were identified and photographed at 250 magnifications in similar adjacent sections of each specimen stained with the different monoclonal antibodies. The photographic slides were then projected on a screen with a superimposed grid, and the labeled cells enumerated by the same observer who was blinded to knowledge of the monoclonal used in staining.

The search for rickettsiae was made by incubating frozen sections, fixed and washed as previously, with a rabbit anti-spotted fever group immunoglobulin FITC-conjugate (CDC, Atlanta, Georgia) reactive with R. conorii for 30 min. in a moist chamber and washing, mounting, and examining as above.

Further studies of pathogenic mechanisms of R. conorii were performed in the plaque model which we have exploited in investigations of pathogenic mechanisms of R. rickettsii. Aliquots of R. conorii stock were thawed and diluted in sucrose phosphate buffer to contain 500 plaque forming units (pfu) per ml. Confluent monolayers of Vero cells were inoculated with either 0.1 ml of diluted rickettsial stock containing 50 pfu of R. conorii or uninfected diluent. Rickettsial plaque technique was performed according to the method of Wike and Burgdorfer and Wike et al. After 30 minutes for absorption to occur and penetration to begin, the monolayer was overlaid with 0.5% and agarose in minimum essential medium with 5% fetal bovine serum and incubated at 35°C. On day 4 after inoculation, 4 ml of second overlay with 1% neutral red was added, and the flasks were allowed to incubate in the dark at 35°C.

Flasks were examined daily for plaques afterwards with observations of monolayers by inverted microscope and with collection of specimens for examination by immunofluorescent and transmission electron microscopy.

The sides of the 25-sq cm Falcon flasks opposite the monolayers were removed by cutting the plastic. Agarose gel overlays were gently removed by separating the overlay from the sides of the flask with a sharp spatula edge and allowing the gel to detach under the force of gravity. Exposed monolayers were fixed in 70% ethanol for 20 minutes prior to direct immunofluorescent staining for R. conorii with a specific anti-R. conorii conjugate. After incubation of monolayers with conjugate for 30 minutes, they were washed in phosphate-buffered saline for 30 minutes, washed in distilled water, and mounted with 90% glycerol in phosphate-buffered saline (pH 9) and cover glass. Monolayers were examined on a Leitz Ortholux ultraviolet microscope equipped with the appropriate barrier, exciter, and edge filters for fluorescein isothiocyanate fluorescence microscopy.

Monolayers with overlays removed as described for immunofluorescence were fixed by covering the cells with a solution of buffered 2.5% glutaraldehyde for 1 hour. Cells were maintained on the plastic surface throughout postfixation in osmium tetroxide, dehydrated through a graded series of ethanol and hydroxypropyl methacrylate solutions, and embedded in Mollenhauer's Epon-araldite No. 2 followed by polymerization in an oven at 37°-45°C for 24 hours and then 60°C overnight. Embedded monolayers were separated from the plastic flasks. At this point, rickettsial plaques were observed with the unaided eye as distinct clear zones surrounded by a grey-black carpet of cells. Plaques and adjacent cells were cut out and reembedded in flat molds with the monolayer perpendicular to the plane of sectioning. Ultrathin sections were cut on an LKB ultramicrotome using a diamond knife. Observation of the block during sectioning reveals the exact relationship of the section to the plaque. Sections were stained with lead citrate and uranyl acetate and examined on a Zeiss 10A transmission electron microscope.

Plaque size, time of appearance and phase contrast morphology were observed. The cytopathology of injured cells was described including state of rough endoplasmic reticulum, mitochondria, plasma membrane, and nucleus. The cytology of uninfected cells adjacent to the plaque was examined.

Plaque model studies of penetration-associated pathogenic mechanism employed the plaque model, phentermine, and indomethacin, compounds which had been shown to have phospholipase inhibitory activity were incorporated into the agarose-nutrient medium overlay. The second overlay contained the same concentration of the drugs. Plaques were enumerated at the time of appearance of distinct plaques in untreated plaque assays of the same inoculum for statistical analysis. In a second set of similar experiments the protease inhbitor, BABIM, was introduced in the overlay.

One hypothesis which was tested in the plaque model was that the paucity of signs and symptoms pointing to visceral involvement was due to a lower threshold of temperature sensitivity of R. conorii. The inability to produce pathogenic effects at temperatures greater than 38°C could explain the relative lack of severity of BF when compared with RMSF despite the 91-94% relatedness of the etiologic agents. The plaquing efficiency of R. conorii was compared at 32°C, 34°C, 36°C, 38°C, and 40°C. Variation in number of plaques formed, and time of onset was examined. These results reflect the effect of temperature on pathogenic effects.

The hypothesis of secretion of a potent extracellular toxin by R. conorii was examined in an experiment utilizing parabiotic tissue culture chambers. Parabiotic chambers containing cell monolayers were separated by a filter with 0.22 μm pore size. This filter prevented the passage of rickettsiae from one

chamber to the adjacent chamber, but allows free passage of macromolecules such as metabolites, putative toxic products, or enzymes. In some pairs of chambers, one chamber was inoculated with R. conorii. Other pairs of chambers were maintained with both chambers uninoculated as controls. On days 3, 5, and 7 postinoculation, trypan blue was added to selected pairs of chambers, and selected chambers were examined by immunofluorescence for R. conorii. The degree of cell injury in infected chambers, uninfected-rickttsial products exposed chambers, and control chambers was evaluated blindly by estimation of percentage of cells failing to exclude trypan blue. Immunofluorescence for R. conorii confirmed the limitation of infection to inoculated chambers and allowed estimation of the percentage of the monolayer that is infected.

Results

The study of taches noires from patients with boutonneuse fever was performed in collaboration with physicians at the University of Palermo. This collaboration has proven successful with opening of several avenues for the continued investigation of the pathology, pathophysiolgoy, and clinical aspects of R. conorii infection in humans. During the period June-August 1983, I spent six weeks in Sicily as NATO Visiting Professor of Tropical and Subtropical Diseases. My major collaborators were Professor Mansueto for clinical studies of boutonneuse fever and Professor Tringali for rickettsial studies. Twenty patients with clinical boutonneuse fever under the care of Dr. Barba at the Guadagna Infectious Disease Hospital were investigated. During the visiting professorship, seven biopsies of taches noires were collected. With four additional tache noire biopsies collected prior to my arrival and after my departure, 11 patients' taches noires were biopsied in all during 1983. Some patients with taches noires were not biopsied principally for reasons of location related to cosmetic conern or proximity to vital structures, e.g., carotid artery. Other patients had boutonneuse fever without a tache noire. Rickettsia conorii was isolated from patients by inoculation of guinea pigs. Intradermal inoculation resulted in formation of an eschar at the site of inoculation in many animals. Guinea pig eschars were demonstrated to contain R. conorii by direct immunofluorescence with the anti-spotted fever group rickettsia conjugate obtained from the Centers for Disease Control.

Four studies of patients with boutonneuse fever in Sicily were initiated prior to the beginning of this research contract. They were completed and prepared for publication during the first year of this project. In the first study, the method for demonstration of R. conorii in infected tissues fixed in formalin and embedded in paraffin utilizing the deparaffinization, trypsin digestion method and immunofluorescence was described. T-lymphocyte deficient, nude mice were inoculated cutaneously with 4.8×10^4 plaque forming units of R. conorii (Malish 7 strain) and were sacrificed on days 3, 7, and 15 after inoculation. An adult guinea pig was inoculated intradermally with the same inoculum and sacrificed on day 11. Skin from the inoculation site, spleen, and liver of the mice and skin from the inoculation site eschar of the guinea pig were collected and fixed in 4% neutral buffered formaldehyde. A biopsy of the rash from a Sicilian patient with boutonneuse fever was obtained on day 6 after onset of fever and was similarly processed. Sections at 4 um thickness were affixed to slides with LePage Bond Fast Resin glue (LePage, Ltd., Montreal, Canada) and were incubated in an oven at 60°C for 1 hr., deparaffinized in three changes of ethanol in concentrations of 100%, 95%, 70%, 50%, 35%, and finally in distilled water. The slides were then incubated for 4 hrs in a 0.1% trypsin (Grand Island Biological Co., Grand Island, NY)

with 0.1% $CaCl_2$, pH 7.8 at 37°C. After incubation slides from the patient and from mice were washed thoroughly in distilled water, washed for 30 min in PBS, and allowed to react for 30 min with anti-R. conorii guinea pig serum prepared in our laboratory, washed for 30 min in PBS, and incubated with specific anti-guinea pig immunoglobulin FITC conjugate (DAKO) for 30 min, washed in PBS for 30 min, rinsed in distilled water, mounted in a solution of glycerol in PBS, and examined by ultraviolet microscopy with use of FITC barrier and exciter filters. For guinea pig tissues we used anti-R. conorii human serum and an anti-human conjugate from DAKO.

Furthermore, we submitted all the slides to similar procedures but using direct immunofluorescence with a rabbit immunoglobulin FITC conjugate prepared against the antigens of R. rickettsii at the Centers for Disease Control, Atlanta, Georgia, as described previously by Walker and Cain.

Organisms with morphology of rickettsiae were observed predominantly in the vascular walls in the endothelial location in clusters or arranged end-to-end. Only smaller quantities were observed away from the vessel walls, apparently associated with the predominantly macrophagic perivascular cellular reaction. These organisms were demonstrated by both indirect immunofluorescence and direct immunofluorescence. An observation deserving comment is the better results obtained with the direct reaction using rabbit anti-R. rickettsii conjugate. Hebert et al have shown that this serum is specific for the spotted fever group of rickettsiae. It is a well known, high-titered, well standardized reagent that gives consistently excellent results. It reacts with R. conorii and the titer with this agent is only one dilution below the titer with R. rickettsii. In our hands it gives good, clean preparation. The indirect test was performed with guinea pig or human anti-R. conorii whole serum, both with relatively low titers. It is known that indirect immunofluorescence gives a certain degree of nonspecific fluorescence. Indeed, in our preparations there was some of this nonspecific fluorescence, and sometimes we had to rely on the location, size, and morphology of the fluorescent structures to consider them to be rickettsiae. In a second publication, the histologic lesions of the tache noire were described and organisms of R. conorii were demonstrated in 5 of the 6 biopsies using the deparaffinization-trypsinization immunofluorescent technique.

One of these patients is described in detail in a case report in the American Journal of Tropical Medicine and Hygiene because of the documentation of the mild end of the clinical spectrum of human R. conorii infection that he represents. A sixty year old agricultural laborer from Sciacca, Italy noted the presence of a tick on the lateral aspect of the right lower leg and removed it on March 10, 1982. Twenty days later he sought medical attention because of the development of a skin lesion at the site of tick bite. The cutaneous lesion consisted of a central, black ulcer 2 cm in diameter surrounded by vesicles and peripheral erythematous zone. Indirect immunofluorescent antibody assay confirmed the diagnosis of BF with anti-R. conorii IgG titer of 1:320 and IgM titer of 1:80.

On April 3, 1982, a biopsy of the tache norie was performed. Brightfield microscopy revealed foci of pseudoepitheliomatous hyperplasia in the epidermis surrounding the necrotic mass of karyorrhectic debris, fibrin and keratin, corresponding to the eschar. In the surrounding dermis and underlying subcutaneous tissue, there were perivascular accumulations of macrophages, lymphocytes, and numerous eosinophils. The vascular endothelial cells were swollen. Examination of sections by the method of deparaffinization and trypsin digestion followed by direct immunofluorescence with a conjugate reactive with spotted fever group rickettsiae showed focal clusters of coccobacillary organisms in the lining of the vessel walls in the reticular

dermis. The immunofluorescent conjugate was prepared at the Centers for Disease Control using killed R. rickettsii as antigen for immunization of rabbits. The conjugate of the globulin fraction of rabbit antiserum has also been demonstrated to react with R. conorii at a titer of 1:512.

Reaction of sections of the eschar with guinea pig preserum by indirect immunofluorescence using a 1:20 dilution of serum and 1:40 dilution of rabbit anti-guinea pig IgG conjugate (DAKO, Accurate Chemical and Scientific Corporation, Westbury, NY) revealed no organisms whereas the same indirect immunofluorescent system using convalescent serum collected from the same animal one month after inoculation of R. conorii revealed foci of thin bacilli compatible with rickettsiae.

On April 6 the anti-R. conorii titers remained at the same level while both the third and fourth components of complement were slightly elevated. At no time during his course did the patient report a fever. He was afebrile and did not have a rash during this evaluation of the eschar or during the following eight months.

The observation of spotted fever group rickettsiae at the site of tick bite is strong evidence for inoculation of rickettsiae into the skin by tick bite, colonization of vascular endothelium by the rickettsiae, and stimulation of host defenses without any systemic signs or symptoms of disease.

The two principal hypotheses that may explain the occurrence of R. conorii infection manifested only by an eschar are either that the strain of R. conorii-like rickettsia was of relatively low virulence or that previous spotted fever group rickettsial infection provided partial immune protection. Investigation of spotted fever group rickettsiae in North America has revealed a great diversity of rickettsial species with a spectrum of virulence as judged by response of guinea pigs to inoculation. At the present time the range of virulence of R. conorii and possibly other spotted fever group rickettsiae in the Mediterranean basin is not known. The serologic documentation of infection with R. conorii among as many as 20% of persons in western Sicily who are engaged in agricultural activities and give no history of BF suggests that there are nonpathogenic strains of R. conorii in Sicily. The possibility of a previous infection with R. conorii cannot be excluded. The serology, in fact, demonstrated a higher level of antibody to R. conorii in the IgG class than the IgM class, as would be expected in an anamnestic immune response. Bourgeois et al have shown that in primary infection with R. tsutsugamushi the antibody response is mainly of the IgM class. In contrast, reinfection scrub typhus stimulates an antibody response mainly of the IgG class. We have also observed these two types of antibody response to R. conroii in BF. It may be hypothesized that our patient, an agricultural laborer who is at high risk for tick bite and/or R. conorii infection, had a prior infection with residual immunity sufficient to contain the subsequently inoculated organisms at the portal of entry. Elucidation of the host-rickettsia relationship between humans and R. conorii will require extensive investigation of strains of R. conorii isolated in Sicily and of the immunology of human host defenses against rickettsiae.

The fourth manuscript on R. conorii infection in vivo prepared using data collected prior to the start of this contract presents a study of infection of genetically immunodeficient mice with R. conorii. In order to determine the definitive importance of T- and B-lymphocytes in immunity to Rickettsia conorii, mice genetically deficient in T-cells, B-cells, or both T- and B-cells were infected experimentally. T-lymphocytes rather than humoral antibodies were crucial to rickettsial clearance and a reduced mortality rate. Mice incapable of an antibody response to polysaccharide capsular antigens effectively controlled rickettsial infection with no mortality. In contrast,

nude mice produced antibody to thymus-independent antigens early in the course of infection, yet experienced severe rickettsial infection with deaths occurring.

These experiments in genetically immunodeficient mice document the importance of T-lymphocytes in the clearance of R. conorii from the tissues of infected mice. On day 7 the spleens and livers of T-cell deficient and T- and B-cell deficient mice contained numerous rickettsiae, whereas animals with an intact T-lymphocyte immune system contained very few rickettsiae in their hepatic and splenic tissues. The only animals that died as a result of R. conorii infection in these studies were those deficient in T-lymphocytes whether or not they had B-lymphocytes. These results support the conclusion of Kokorin et al that T-lymphocytes confer protection against R. conorii infection in mice. Moreover, the generation of a humoral immune response in our experiments did not correlate with clearance of rickettsiae and protection from death. T-lymphocyte deficient nude mice synthesized antibodies to R. conorii detectible on day 7 at which time the visceral organs contained many rickettsiae. Despite the presence of antibodies in these animals, mortality was observed. Nevertheless, B-lymphocyte deficient mice had no antibodies and yet had effectively restricted rickettsial proliferation in the liver and spleen on day 7; moreover, none of these mice died. These observations conform to the conclusion of previous studies on immunity to members of the genus Rickettsia, namely that T-lymphocytes are crucial for immunity.

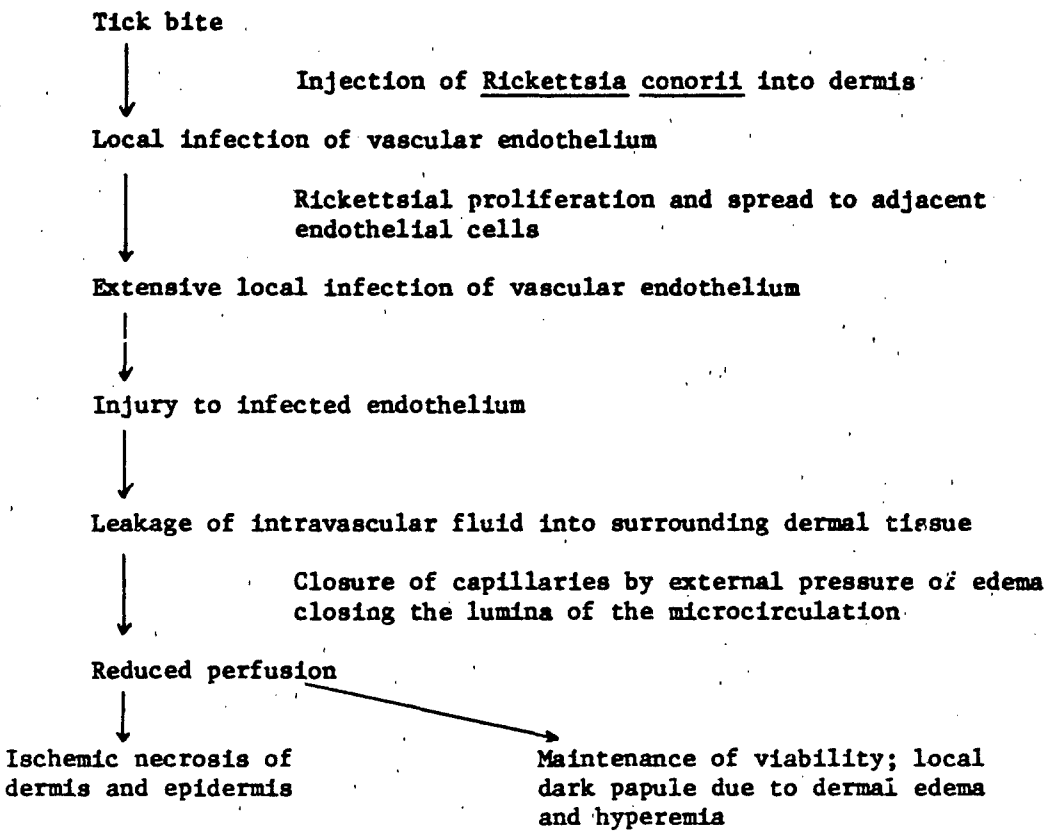
The immune response has not been shown in this or previous studies to be a pathogenic mechanism of tissue injury in infections by members of the genus Rickettsia. The cytolytic effect of lymphokines on rickettsia-infected cells in vitro again raises the question of an immunopathologic mechanism of tissue and cellular injury in rickettsial diseases. Although this investigation does not exclude a contribution to cell injury by lymphokine-mediated cytolysis, it does document that in the overall balance in vivo the T-lymphocyte affords protection against R. conorii. Other studies including the in vitro plaque model have demonstrated that rickettsiae possess direct cytopathic activity that appears to be mediated at least in part by the phospholipase- associated penetration mechanism and is active in the absence of the immune system.

The similarity of the hepatic lesions of these animals to hepatic lesions in boutonneuse fever contributes to understanding of the pathogenesis of the human lesions of multifocal hepatocellular necrosis and associated focal hepatic inflammatory response. The observation of R. conorii in these lesions in mice suggests that the rickettsiae play an important role in their pathogenesis. The immunodeficient mouse model of R. conorii infection should be useful in further dissection of pathogenic mechanisms of hepatic injury by R. conorii and in evaluation of the importance of stimulation of each component of the immune system by specific purified rickettsial antigens in protective immunity and vaccine design.

By the end of the three year contract period, of biopsies of lesions compatible with tache noires from 24 patients in Sicily, 16 have been documented as BF, 1 was shown not to have BF, and 7 have incomplete data at present. Study of the documented cases semiquantitatively for presence and severity of specific pathologic features (Table 1) yielded the following: cutaneous necrosis was present in 10 of 15 evaluatable tache noires; vasculitis was severe or moderate in all 16; thrombosis was severe in only 1, moderate in only 1, mild in 4, and absent in 10; dermal edema was moderate in 12, mild in 4. The predominant leukocytes were lymphocytes and macrophages; immunofluorescent Rickettsia conorii were demonstrated in 12.

These results indicate that vascular injury by rickettsiae is the major lesion and that dermal edema is the important result. Thrombosis was generally absent or only focal and mild. Thus, the pathogenetic sequence is likely to be:

PATHOGENETIC SEQUENCE OF EVENTS IN THE TACHE NOIRE



Tissues from ten tache noires (seven from Palermo and three from Marseille) have been embedded for transmission electron microscopy and examined by 1 micron toluidine stained sections. Blocks from seven patients were considered suitable for thin section ultrastructural study. These sections revealed severe necrosis, fibrinous exudates, and mononuclear cellular infiltration. Rickettsiae were rarely observed.

In immunohistochemical evaluation of the Spanish taches noires, histological examination of the sections showed in five the typical lesions described in the tache noire: epidermal ulceration, variable degrees of coagulative necrosis and edema in the upper dermis, diffuse vascular alterations characterized by vasodilation, endothelial swelling and edema of the vascular wall, intense perivascular inflammatory infiltrate, and occasionally nonocclusive thrombosis. One case lacked epithelial ulceration. The inflammatory infiltrate was formed predominantly by mononuclear cells which surrounded and invaded the wall of small vessels around the zone of central necrosis.

The number of enumerated cells which showed positive stain with the monoclonal antibodies, anti-leu-4, anti-leu-12 or anti-leu-M3 in each specimen was 1202 $\pm$ 357 ($x \pm SD$). Table 2 shows the results of the counts together with other data regarding the patients and the sample studied. The cell populations, leu-4^+, leu-12^+ and leu-$M3^+$, are expressed in terms of percentage of the total of counted cells and the number of cells with leu-$3a^+$ and leu-$2a^+$: 55.71 $\pm$ 6.7%; leu-12^+:17.66 $\pm$ 4.65%; leu-$M3^+$: 26.64 $\pm$ 5.02%; and leu-$3a^+$/leu-$2a^+$ ratio 1.399 $\pm$ 0.127. No particular microscopic pattern of distribution was observed in the analyzed perivascular cellular populations.

Five of the samples contained immunofluorescent rickettsiae. Numerous immunofluorescent coccobacillary organisms were identified, isolated or in groups, spread through the zone of necrosis or in blood vessels with perivascular inflammatory infiltrates.

Our quantitative evaluation of the mononuclear cell populations in perivascular inflammmatory foci of the tache noire suggests the importance of T-cell mediated immunity in the local host defense against R. conorii.

Enumeration of the T-cell subsets in our model showed the relatively more numerous presence of T-helper/inducer cells which are known to be the source of mediators that enhance the immune response. Among the lymphokines, gamma-interferon seems to be the predominant factor which interacts with macrophages to inhibit rickettsial growth or to kill rickettsiae and interacts with fibroblasts and endothelial cells to inhibit the organisms.

A specific T cell mediated cytotoxic mechanism has been recently demonstrated acting against murine fibroblast cells infected with R. typhi and R. tsutsugamushi. This phenomenon is congruent with the expression of rickettsial antigens on the surface of infected cells. However, the importance of the T cell cytotoxic subset in infection with SFG rickettsiae is actually not known at present. Its postulated role in the pathogenesis of these diseases by action on endothelial cells is contrasted with the actual knowledge that rickettsiae themselves are able to cause the cellular damage probably responsible for vascular permeability, platelet aggregation and other pathophysiological effects.

Studies that have shown macrophages to be crucial effectors of immunity have employed models of infection in which rickettsiae are inoculated by the intraperitoneal route and peritoneal macrophages are the major target cell. It should be noted, however, that this is hardly the situation in naturally occurring disseminated endothelial infection. Macrophages may share with endothelial cells the work of presenting rickettsial antigens, but macrophages

themselves are probably more competent as effectors when they do encounter rickettsiae.

The identification of rickettsiae in taches noires has been employed as a diagnostic test in MSF. The only case in which we failed to identify microorganisms corresponded to sections that did not contain the central necrotic area of the specimen. In two of four biopsies of rash examined simultaneously with taches noires, we also failed to demonstrate rickettsiae. They both were obtained late in the course of the disease (days 11 and 17) from patients with IF antibody titers greater than 1:320. In all cases, the number of identified organisms correlated with the intensity of the observed lesions. These results emphasize the importance of selection of the tissue sample when using this diagnostic method in addition to previously described factors that reduce the number of rickettsiae observed.

A manuscript on the acute phase reaction in boutonneuse fever has been prepared and submitted for publication. This work was performed in Palermo in collaboration with the Clinic of Tropical and Subtropical Diseases and the Institute of Hygiene. Evaluation of specific serum proteins from 44 patients in Sicily with boutonneuse fever revealed that C-reactive protein, haptoglobin, alpha-1-antitrypsin, C3, and C4 are elevated at the time of presentation for medical attention in varying proportions of the patients. C-reactive protein is invariably elevated as a consequence of active injury and inflammation during the first week of illness. These increased serum protein concentrations follow the pattern of the acute phase reaction and suggest that immunopathologic phenomena and intravascular hemolysis do not occur in most patients with boutonneuse fever.

The haptoglobin concentration was significantly higher during the first two weeks of illness than subsequently. Only three determinations had values below the normal range, one during the first week of illness and two during late convalescence. Serum alpha-1-antitrypsin levels were highest during the first two weeks of illness and had diminished to normal levels by 4 weeks after onset of illness. Concentrations of C3 convertase and C4 were elevated acutely with 16/25 measurements of C4 and 8/25 measurements of C3 convertase above the reference interval during the first week and 20/26 assays of C4 and 5/26 assays of C3 convertase above the reference interval during the second week. In only one patient during the first week of illness were the concentrations of C3 convertase and C4 below the reference interval. C-reactive protein (CRP) was at or above the upper limit of normal in all patients during the first week and in 20/26 patients during the second week. By late convalescence 1-2 months after onset, 15/20 patients had no detectible serum CRP.

This study has demonstrated that the acute phase reaction occurs during boutonneuse fever. Serum concentrations of certain hepatic synthesized proteins are elevated as a sterotyped response following surgical operation, several acute infections, and other situations that have acute inflammation or tissue necrosis. CRP concentration has been shown to rise from undetectible levels within 4-6 hours after acute injury. Haptoglobin, fibrinogen, and alpha-1-antitrypsin are increased in concentration by 24 hours after injury, and C3 convertase is elevated during the first week. Our measurements were not made early enough in the course of BF to determine how many hours after onset were required before the rise occurred.

The effects of the elevated concentrations of these serum proteins on the pathophysiology of BF is uncertain. It may be hypothesized that C3 convertase and C4 might be consumed by binding to circulating immune complexes, which have been demonstrated to occur in BF. Although the observation that C3 convertase and C4 concentration are mostly elevated or

normal does not exclude that possibility entirely, it does not support that hypothesis. Other observations and experimental data suggest that immunopathologic mechanisms are not important in spotted fever group rickettsioses. Nonspecific opsonization of spotted fever rickettsiae by direct reaction with components of complement or CRP has never been demonstrated; however, elevated concentrations, particularly of CRP, suggest this as a possible host defense mechanism as it may be in other bacterial infections. The persistent elevation of CRP in some patients indicates that tissue injury or inflammation is ongoing.

The observation of a low plasma concentration of haptoglobin in one patient during the acute phase of BF indicates that significant hemolysis is not a frequently occurring complication. The recent demonstration of hemolysis in a patient with glucose-6-phosphate dehydrogenase (G6PD) deficiency and fatal RMSF suggests that hemolysis may play a role in the enhanced severity of RMSF in G6PD deficient men. Since males with G6PD Mediterranean have been shown to have a higher incidence of specific complications in BF than G6PD normal males, the possibility of hemolysis should be now evaluated in this subpopulation of patients with BF.

In general, the acute phase reaction appears to be an immediate response which response if highly likely to have resulted from evolutionary selection and to have an effect that is generally favorable to the host. The exact interactions of C3 convertase, C4, and CRP with R. conorii await further study.

The data on BF indicate that R. conorii is not merely a cutaneous infection in humans. This fact was documented by examination of hepatic biopsies in seven Sicilian patients and demonstration of hepatic lesions in each of them. During 1983 and 1984, seven patients supected of having BF consented to hepatic needle biopsy. The clinical and serologic data supporting the diagnosis of R. conorii infection are presented in Table 3. Two patients had a four-fold rise in titer of antibodies to R. conorii documented by indirect immunofluorescent antibody assay. All seven had serum antibodies to R. conorii; five patients at a titer of 1:160 or higher. A tache noire and fever were observed in all seven patients; a rash, in five patients. Thus, the diagnosis of BF can be considered as confirmed in two patients and very probable in the other five. None had signs or symptoms indicative of hepatic disease. Data on serum concentrations of lactate dehydrogenase, alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl tranpeptidase, and bilirubin and results of evaluation for presence of hepatic lesions and R. conorii are presented in Table 4.

The moderate elevations of serum concentrations of LDH, AST, and ALT reflect the presence of scattered foci of hepatocellular necrosis, which involved only a small porportion of hepatocytes. The lobular location of these lesions and the absence of involvement of portal triads is reflected in the absence of striking deviations of laboratory measurements for serum bilirubin and alkaline phosphatase during the acute phase of the illness. Nevertheless, it is remarkable that all biopsies contained lesions which appeared to fit into the sequence of hepatocellular necrosis followed by focal, predominantly mononuclear leukocytic, inflammatory reaction. Yet, in no case were intact immunofluorescent SFG rickettsiae identified in the tissue. This investigation confirms previous reports of hepatic lesions in patients with BF. It is our interpretation that these lesions do not fit the designation granulomatous hepatitis, but rather consist of foci of hepatocellular necrosis and predominantly mononuclear leukocytic reaction to the necrosis and probable former site of R. conorii infection. The lesion differs from a true granuloma in that it is not an avascular

aggregate of activated macrophages in contrast to the granulomatous hepatitis of Q fever in which aggregates of macrophages are observed in a peculiar doughnut arrangement. C. burnetii resides within the phagolysosome of macrophages, whereas endothelial cells are the primary target of R. conorii in most organs. The target cell of R. conorii in liver awaits further study of human cases and animal models. In fatal cases of R. conorii infection in South Africa, immunofluorescent rickettsiae have been demonstrated in hepatic sinusoidal lining cells that may have been Kupffer cells or endothelial cells. Adjacent necrosis was associated with foci of rickettsial infection in those fatal cases, thus suggesting a role for rickettsiae in hepatocellular injury and the probable clearance of rickettsiae from lesions in the biopsies by effective host immune and phagocytic mechanisms.

These lesions resemble multifocal hepatocellular necrosis and inflammation observed in mice infected experimentally with R. conorii (84). The observations that similar lesions occur in both immunocompetent and T-lymphocyte deficient mice, although more rickettsiae persist in the immunodeficient mice, suggest that immunopathologic mechanism are not important in the pathogenesis of these lesions. Likewise, the fatal South African cases contained necrotic hepatic cells in foci that did not contain a cellular response. Future studies of human and experimental animal material by electron microscopy and immunohistochemistry will be important for characterization of the populations of inflammatory cells and subpopulations of T-lymphocytes as well as for identification of the hepatic target cell of R. conorii.

Finally, the most important conclusion of the human study is that BF must not be considered as a benign disease with rare extracutaneous involvement. The demonstration of hepatic lesions in seven consecutive patients evaluated by liver biopsy suggests that R. conorii is frequently viscerotropic and in patients with particular risk factors poses a serious threat.

Moreover, the clinical spectrum of BF must be expanded to include a malignant form that often results in death. This situation has been reported in France, South Africa, Israel, Spain, Belgium, and Portugal. Old age, alcoholism diabetes, male sex, and glucose-6-phosphate dehydrogenase deficiency appear to predispose to a severe course. The severe form of BF is characterized by cutaneous purpura, neurologic signs, respiratory symptoms, hepatomegaly, thrombocytopenia, acute renal failure, and elevated concentration of serum enzymes such as creatine kinase. Hemorrhagic manifestations include petechial or purpuric rash, epistaxis, metrorrhagia, and gastrointestinal hemorrhage, the latter of which may be fatal. This form of the illness is not rare. It has been reported in 8% of 71 cases from the Salamanca (Spain) between 1981 and 1984, in 3% of 154 cases from Marseille between 1974 and 1981, in 4 patients in Marseille during the two years 1981-82, in 5% of 142 patients in Marseille during 1983-84, and in other case reports. The fatality rate of BF may, in fact, be nearly as high as the 5% rate currently observed for Rocky Mountain spotted fever in the US. The fatality rates for recent series include 5.6% of 71 patients in Salamanca, 3% of 63 patients in the Negev, 2% of 154 patients in Marseille during 1974-1981, and 1.4% of 142 patients in Marseille during 1983-84.

The occurrence of hemolysis and severe BF has been documented in a previously healthy 27 year old man with G6PD deficiency by the Infectious Diseases Unit of the Boigny Hospital and the Pediatric Hematology Unit of La Timone Hospital in Marseille. A 27 year old previously healthy Algerian man was hospitalized on August 3, 1984, after two days of fever,

headache, myalgias, asthenia and weight loss of two kilograms. Several days beforehand he had noticed a scrotal lesion with painful inguinal lymphadenophathy. On admission he had temperature 39.5°C, pulse 100/mn, blood pressure 110/60 mmHg, and scrotal eschar. He became stuporous and confused. A maculopapular eruption involving the palms and soles and purpuric on the legs and trunk was observed on August 7, 1984. The diagnosis of BF was made, and intravenous treatment with doxycycline, 200 mg/day, was begun on August 7. On August 10, 1984, the patient was still stuporous but afebrile. Laboratory data showed hepatic involvement (SGOT, 145/ IU/l and SGPT 142 IU/l) and evidence of hemolysis with decreased hemoglobin and haptoblobin but normal bilirubin. The subsequent course was uneventful and did not require blood transfusion. G6PD phenotype was determined to be G6PD B$^-$ by low G6PD activity determined by spectrophotometry and by electrophoretic mobility. The diagnosis of BF was confirmed by IFA serology. Investigation of the family revealed two other cases of G6PD deficiency, a brother and a maternal uncle. G6PD deficiency, suspected to be a risk factor for severe disease, occurs in southern France especially in Marseille with its population of diverse origins including Corsica and North Africa. G6PD deficiency has previously been associated with severity in murine typhus, scrub typhus and Rocky Mountain spotted fever. Regarding BF, Meloni and Forteleoni found no statistical differences in patients with and without G6PD deficiency although no particular criteria were stated. In contrast, Piras et al reported an evaluation of Sardinian males with BF for complications of particular organ systems which occurred more frequently in G6PD deficient patients. Neither study commented on examination for hemolysis.

Raoult's patient had a quite severe form of serologically confirmed BF with stupor, confusion, purpuric exanthem, thrombocytopenia, hepatic involvement, and evidence for hemolysis. The hemoglobin fell from 14.7 g/dl to 9.9 g/dl, and serum haptoglobin was depleted (0.16 g/l) acutely and rose to normal concentration (1.5 g/l) during convalescence. This is the first reported case of hemolysis in a G6PD deficient patient suffering from BF and the first reported case of hemolysis with rickettsiosis in a G6PD B$^-$ (Mediterranean) patient. The mechanism of enhanced severity of rickettsioses in G6PD deficiency is unclear. Three hypotheses are drug induced hemolysis, increase of rickettsial activity due to defective host defense, or stimulation of rickettsial virulence by constituents released from lysis of red blood cells. In this case hemolysis occurred before tetracycline treatment. This man was the only patient with documented G6PD deficiency among 49 males with BF studied in Marseille. Furthermore, he is the only patient under 30 years of age to have the severe form of the disease.

Examination of needle biopsies from four patients sent by Dr. Faure in Grenoble, France, who has reported the hepatic histopathology of boutonneuse fever, also revealed no immunofluorescent R. conorii.

Because of reports of severe human R. conorii Spain, Belgium, and Israel, I corresponded with three of the authors. This resulted in the opportunity to evaluate the pathology of severe and fatal R. conorii infection including three necropsies. The pathology and distribution of R. conorii in fatal cases has not been reported previously. Dr. Gear, an eminent senior rickettsiologist from South Africa, reported two fatal cases and a severe case with digital gangrene. He has shipped to our laboratory material from the gangrenous digits, from the necropsy of one of the fatal cases, and from the necropsy of a subsequent fatal case, all documented as R. conorii infections. Drs. Ruiz and Herrero from Spain sent tissues from a fatal case of Mediter-

ranean spotted fever. Dr. Raoult from France did not have necropsies on his cases.

Paraffin sections of tissues from the three cases of South African tick bite fever were stained by routine hematoxylin-eosin method, phosphotungstic acid-hematoxylin technique, and deparaffinization-trypsin digestion-immunofluorescence method with a conjugate reactive with R. conorii as reported in detail previously. Tissues examined were cerebrum, cerebellum, kidney, liver, spleen, heart, lung, lymph node, and adrenal gland (case 1); cerebrum, kidney, liver, spleen, heart, lung, and pancreas (case 2); and gangrenous fingers (case 3). Positive control tissues for R. conorii immunofluorescence included eschar from guinea pig inoculated intradermally with R. conorii (Malish 7 strain), eschars from patients with serologically documented boutonneuse fever, and liver tissue from nude mouse inoculated with R. conorii (Malish 7 strain).

Thin bright green immunofluorescent bacilli were observed only in the location of vascular endothelium and of macrophages in hepatic and splenic sinusoids and lymph node sinuses. Nonspecific staining of host tissues was not seen.

In case 1 the brain contained numerous rickettsiae in cerebral and cerebellar blood vessels and foci of rickettsiae in the subarachnoid location of the leptomeninges. Rickettsiae were observed in glomerular arterioles, capillary tufts, intertubular blood vessels, and arterial endothelium. In the liver rickettsiae were identified only in a few sinusoidal lining cells; no rickettsiae were seen in hepatocytes. Splenic rickettsiae appeared to be in macrophages and arteriolar endothelium. Few foci of R. conorii were observed in capillaries between myocardial cells, pulmonary alveolar septa, and macrophages within marginal and draining sinuses of a lymph node. No rickettsiae were demonstrated in adrenal gland.

In case 2, numerous rickettsiae infected cerebral blood vessels, glomerular arterioles and capillary tufts, renal arterial endothelium, and intertubular blood vessels near the corticomedullary junction. Few hepatic rickettsiae were observed in scattered sinusoidal lining cells. In spleen, rickettsiae were identified in arteriolar endothelium and macrophages in small quantities and in one medium sized artery in a large amount. In the heart, a few foci of rickettsiae were present in arterial endothelium and capillaries between myocardial fibers. Very few rickettsiae infected pulmonary alveolar capillaries. Small foci of rickettsiae were seen in capillaries and septal blood vessels of the pancreas and blood vessels in the dermis of skin.

In case 3, several foci containing numerous R. conorii were identified in the injured blood vessels at the margin between viable and necrotic tissue. Rickettsiae were not observed in the mummified necrotic zone or in the zone of healthy tissue.

In case 1 the cerebrum and cerebellum contained numerous foci of perivascular mononuclear cells, some of which infiltrated the adjacent neuropil. The subarachnoid space had a mild infiltration of mononuclear cells and a focal small hemorrhage with erythrophagocytosis by macrophages. The kidney was severely autolytic, but multiple foci of mononuclear leukocytic vasculitis could be identified in the outer part of the medulla near the corticomedullary junction. Hepatic lesions included multifocal, randomly distributed coagulative necrosis of solitary hepatocytes, few polymorphonuclear leukocytes and moderate quantities of small lymphocytes in portal triads, mild steatosis, moderate congestion, and mild sinusoidal leukoyctosis. There were no granulomas, portal vasculitis, or leukocytic accumulation around necrotic hepatocytes. Matching of serial sections by

brightfield microscopy and immunofluorescence demonstrated necrotic hepatocytes adjacent to infected sinusoidal lining cells and R. conorii within splenic arterioles that contained thrombi and karyorrhectic debris. The red pulp was congested, and no germinal centers were observed. The myocardium contained a few foci of interstitial leukocytes, predominantly mononuclear cells. Lung lesions included protein-rich pulmonary edema, congestion, focal nonocclusive thrombosis, focal acute pneumonia with alveolar polymorphonuclear leukocytic exudate, and foci of fibrosis containing carbon pigment and birrefringent silica crystals. Lymph node also contained anthracosilicotic fibrosis. There were foci of adrenocortical necrosis with surrounding leukocytic response, and two periadrenal arteries had foci of acute vascular wall necrosis.

In case 2, many foci of vasculitis in the brain consisted of mononuclear leukocytes infiltrating the blood vessel wall and surrounding neuropil. There was also a mild mononuclear leukocytic leptomeningitis. Lesions in the severely injured kidney included karyorrhexis, thrombosis, and leukocytic infiltration of glomerular arterioles, karyorrhexis in glomerular capillary tufts, cortical vasculitis with perivascular mononuclear leukocytes, plasma cells, and focal hemorrhage, multifocal cortical interstitial edema, and multifocal severe vasculitis with petechiae at the corticomedullary junction and in the outer part of the medulla. The liver contained multifocal necrosis of solitary hepatocytes, intracanalicular cholestasis, mild steatosis, hepatocellular giant cell transformation, and mitosis. No granulomas, portal vasculitis, or leukocytic response to necrotic hepatocytes were observed. The spleen had a capsular hemorrhage and polymorphonuclear leukocytes and plasma cells in the red pulp. The lungs were congested with intraalveolar amorphous eosinophilic material compatible with pulmonary edema, scattered intraalveolar erythrocytes, and deposits of carbon pigment. The pancreas was autolyzed, but contained a focus of identifiable perivascular mononuclear cell infiltrate. The skin showed multifocal dermal and subcutaneous vasculitis with perivascular edema and leukocytes including polymorphonuclear leukocytes. Two dermal blood vessels contained thrombi that did not occlude the lumina.

A 77 year old woman from a rural area near Salamanca, Spain, developed a febrile disease which ran a fatal course of 18 days. She had a past medical history of hypertension, adult onset diabetes mellitus, and moderate dyspnea on exertion. Sixteen days prior to admission she developed fever, chills, nonproductive cough, increased dyspnea, anorexia, headache, and myalgias. On the fifth day of illness empiric treatment with amoxicillin was begun. When a generalized rash was observed on the ninth day of illness, it was thought to be a drug reaction. Fever persisted and her general condition deteriorated. On admission to the university hospital of Salamanca, she was noted to have a fever (39.5°C), tachycardia (128/min), hypotension (100/60 mm Hg) with no signs of congestive heart failure, generalized purpuric maculopapular rash involving also the palms and soles, eschar in the left axilla, tachypnea, rhonchi, crepitant bibasilar rales, right upper quadrant abdominal tenderness, edema of the lower extremities, confusion and stupor. Laboratory data included WBC 28,300/ul with 75% PMN's, 12% bands, 3% lymphocytes, and 7% (0.07) monocytes, platelets 127,000/ul, BUN 213 mg/dl, LDH 780 U/L, AST 53 U/L, ALT 27 U/L, GGT 59 U/L, alkaline phosphatase 118 U/L, albumin 2.39 gm/dl, pH 7.50, pCO_2 32 mmHg, pO2 48 mmHg, urine sodium 10 mEq/L, negative blood cultures, and indirect immunofluorescent antibody titer for R. conorii 1:640.

At necropsy significant macroscopic observations were maculopapular rash, left axillary eschar, edema of the legs, acute pneumonia, pulmonary edema, emphysema, atelectasis, and gastric hemorrhage from superficial ulcers in the body and antrum of the stomach.

Microscopy revealed disseminated vascular lesions with prominent perivascular lymphohistiocytic infiltrates in the central nervous system, lung, heart, kidney, esophagus, stomach, colon, pancreas, spleen, adrenal cortex, and thyroid. Focal associated thrombi were identified in lung, heart, kidney, esophagus, stomach, pancreas, spleen, and thyroid. Petechiae were observed microscopically in adrenal cortex and gastric mucosa and submucosa. Other histopathologic lesions included mild-to-moderate interstitial mononuclear myocarditis without necrosis, mild interstitial pneumonia, multifocal hepatocellular necrosis with mixed mononuclear and polymorphonuclear leukocytic infiltrates, active portal triaditis, moderate steatosis, sinus erythrophagocytosis in lymph node, gastric ulceration, and acute bronchopneumonia.

Examination of sections by direct immunofluorescence with a conjugate reactive with R. conorii according to the method of deparaffinization and trypsin digestion revealed no spotted fever group rickettsiae.

The diagnosis of Mediterranean spotted fever was well documented in this patient. The classic signs of fever, rash, and eschar were observed; the epidemiology of rural exposure and summer occurrence was typical. The histopathology was characteristic of disseminated rickettsial vasculitis, and a specific serologic test demonstrated a high titer of antibodies to R. conorii. The clinical course was severe not only as a result of the fatal outcome but also because stupor, purpuric exanthem, thrombocytopenia, acute renal failure, pneumonitis, hypoxemia, dyspnea, edema and hypoalbuminemia, some of which have been associated previously with severe MSF, were present.

The microscopic lesions are comparable to those of Rocky Mountain spotted fever (RMSF) and other available descriptions of R. conorii infection. Both this case of fatal MSF and fatal cases of RMSF have disseminated rickettsial vascular infection, injury, and perivascular host mononuclear inflammatory response involving the CNS, gastrointestinal tract, pancreas, heart, lung, and kidney. The necropsy observations in two fatal cases of South African tick bite fever included a similar distribution of rickettsial vasculitis although

our Spanish case of MSF differed in having more severe thrombosis in foci of vasculitis, focal adrenocortical necrosis, and vasculitis identified in the thyroid gland and gastrointestinal tract. The lesions in the CNS and kidney resembled those described previously for MSF in the necropsy of a 20 day old newborn child and a renal biopsy from a 77 year old man, respectively. The hepatic lesions comprised both lobular foci of mononuclear cell inflammation as described previously in hepatic biopsies of patients with uncomplicated MSF and multifocal hepatocellular necrosis without mononuclear cellular response as was observed in the fatal South African cases. The observations from our case, thus, expand the knowledge of the pathologic effects of MSF as well as confirming its similarity to other reported spotted fever rickettsioses.

Likewise, the pathophysiology of this case of malignant MSF correlates well with the observed pathologic lesions and the general pattern of the pathophysiology of rickettsioses. Vascular injury resulted in increased vascular permeability with edema of the legs and lungs, loss of intravascular volume with dehydration, hypoalbuminemia, and prerenal azotemia, rickettsial encephalomyelitis with stupor, rickettsial hepatitis with right upper quadrant abdominal tenderness and elevated serum concentrations of hepatic enzymes, gastrointestinal and pancreatic lesions with anorexia, pneumonitis due in part to rickettsial vasculitis and interstitial pneumonia with cough and hypoxemia, and thrombocytopenia apparently due to consumption of platelets in widely distributed foci of thrombotic vasculitis. The patient has some of the previously reported risk factors associated with severe MSF including old age, diabetes mellitus, and initiation of treatment after the seventh day of symptoms. Other associated factors which this patient was not known to have were alcoholism, heavy smoking, cardiac or respiratory insufficiency, and glucose-6-phosphate dehydrogenase deficiency. In a case of MSF in which an autopsy has been performed, it is possible to address the question of the cause of death. The most remarkable lesions were the disseminated rickettsial vasculitis which may have played a role in the gastric hemorrhage as has been reported previously, pneumonitis and pulmonary edema, and altered mental status that may have led to atelectasis and bronchopneumonia. The progressive hypoxemia was most likely a result of the combination of respiratory lesions (pulmonary edema, vasculitis, bronchopneumonia, emphysema, and atelectasis) and blood loss from gastric hemorrhage.

The question as to why no rickettsial organisms were detected in this patient at autopsy might be explained by the effects of antirickettsial treatment with tetracycline for 30 hours prior to death and the immunity to R. conorii that had developed over the 18 days of illness and unknown incubation period. Nevertheless, there had not been enough time for repair and resolution of the lesions that the rickettsiae had caused and, thus, the fatal complications ensued.

A manuscript on familial cases of boutonneuse fever has been prepared and submitted for publication in collaboration with the same groups of investigators in Sicily. Pairs of cases of boutonneuse fever occurred in three families. The illness appeared nearly simultaneously in both members of the family, but generally was more serious in one as judged by clinical and laboratory parameters. The possibility of a "bed rickettsiosis," that is, reactivation of rickettsiae by the blood meal obtained from the first individual by the same tick which fed upon the second individual, can be excluded in two of the three pairs of cases. Four of the six patients were sleeping in different beds.

On the other hand, the differing severity may be reflected in the different immunological patterns of the two patients. In one couple it was documented that the first and more seriously ill had antibodies of the IgM

class, presumably a result of the first exposure to Rickettsia conorii. The second and less ill patient had antibodies of the IgG class only, presumably the result of reexposure after previous asymptomatic infection with a spotted fever group rickettsia.

The cases reported here, particularly the two sisters, comprise an interesting example from the point of view of clinical and laboratory observations. Clinically, the course was remarkably more severe, as judged by meningeal signs, and more extensive hemorrhagic rash, hepatomegaly, lymphadenopathy, in the younger (54 year old) sister who did not have a significant prior medical history in comparison with her sister (74 year old with a four year history of hypertension). In general, rickettsioses have been documented to be more severe in older patients and clinical judgement would suggest in those with underlying medical problems. Also the laboratory data demonstrated more marked abnormalities in the first of the two patients to become ill.

The hypothesis that a single source of infection, that is to say the same tick, first bit the older sister and then the younger sister with progressive "reactivation" of the rickettsiae after the first blood meal of the tick does not seem very probable. This hypothesis has been propsosed in several cases of Rocky Mountain spotted fever occurring in married couples, familial rickettsioses of the bed, but this proposition is not supported by our cases since the two sisters, although living in the same house, had separate bedrooms.

It seems to us instead more probable that the explanation has an immunologic basis supported by the course of development of immunofluorescent antibodies of a particular class. The younger sister had predominantly IgM antibodies early in her course, and her sister had IgG antibodies. This suggests that the more severe case encountered infection with R. conorii for the first time while the milder case had an anamnestic immune response with rapid rise in the IgG class of antibodies, possibly an indication of previous asymptomatic infection with a spotted fever group rickettsia. This immediate immune response, therefore, allowed a faster antibody rise, and thus, better control of the pathogenic events. It is clear that this hypothesis goes beyond the documented evidence; however, we recently observed a typical "tache noire" (in which biopsy the presence of R. conorii was demonstrated by imunofluorescence) in a patient who did not develop subsequent illness. Meanwhile, monitoring of the immune response showed seroconversion of only IgG antibodies. This observation supports the hypothesis that preexisting asymptomatic infection allowed an anamnestic immune response sufficient to circumscribe the infection at the site of inoculation.

Also in this report are two pairs of cases that appear to document a dynamic analogue. Indeed, in the subjects with a predominant initial IgG response the illness was less severe. The role of humoral immunity in the pathogenesis of rickettsial diseases is not completely clear; nevertheless, several experimental studies have demonstrated a role for antibodies in an eloquent manner. Gambrill and Wisseman have observed that human immune anti-R. prowazekii serum augments the opsonizing power and the destruction of rickettsiae by leukocytes and macrophages. Topping demonstrated protection by prophylactic administration of immune sera. The military significance of these observations is that there are probably "hot spots" of R. conorii prevalence in the ecosystem into which nonimmune trooops might enter and become infected with a substantial attack rate for the group.

A manuscript has been published on the epidemiology of boutonneuse fever in western Sicily that correlates the distribution and prevalence of

R. conorii infection in Rhipicephalus sanguineus with canine seropositivity and human cases of boutonneuse fever. The distribution and prevalence of spotted fever group rickettsial infection in the ixodid dog tick Rhipicephalus sanguineus were found to occur at a rate of 19.7% with variation related to geographical and socioоccupational factors. A higher rate of infection was demonstrated in ticks removed from dogs associated with documented cases of boutonneuse fever.

Out of the 1078 ticks removed from dogs, R. sanguineus was predmoninant with few other species (4 Ixodes ricinus, 4 Hyalomma marginatum, 2 R. bursa). All stages of development were obtained from sheep-dogs, while only adults were observed in domestic dogs living in urban areas and, in late summer, in dogs of the mountainous areas. Only adult ticks were tested in this study. In each place the occurrence of BF has been documented.

A total of 212 (19.2%) of 1078 ticks was found infected with SFG rickettsiae. Not all the areas investigated, however, showed the same rate of infection but a gradient was observed which increased from urban (Palermo, Trapani, Agrigento) to rural areas (Carini, Castellammare, Vicari, Santo Stefano di Quisquina, Alcamo) and decreased again with a rise in altitude (Petralia and the Madonie Mountains). Ticks obtained from dogs living in sheepfolds in rural areas frequently contained SFG rickettsiae; 23/24 (95.8%) of these dogs were found to harbour infected ticks, and all of them were seropositive. In urban areas 14 (43.7%) of 32 dogs were free of infected ticks ($P<0.01$) although antibodies reactive with R. conorii were detected in 25/32 (78%). Higher percentages of dogs carrying noninfected ticks and seronegative dogs were found in mountainous areas: 14/21 (67%) and 10/21 (47%), respectively ($P<0.001$). Seventy-three dogs (67.6%) were found to harbour infected ticks overall. Antibodies reactive with R. conorii were present in 88/108 (83%); yet only 20 of 35 (57.1%) dogs with uninfected ticks were seronegative ($P<0.001$).

Investigation of 8 human cases of BF where association with dogs was documented revealed that 16 out of 22 (73%) dogs tested harboured ticks infected with R. conorii as demonstrated by direct examination of the ticks and seroconversion of guinea pigs. All the dogs were found to have anti-R. conorii antibodies. It is intriguing to note that the case from Santo Stefano di Quisquina was a shepherd and the patients in Castellammare and Vicari, a school teacher and a grocer, respectively, had a sheepfold very near the land surrounding their homes. Controls were chosen and taken from the same neighborhood from houses where no cases of BF had occurred; a significantly lower percentage of seropositive dogs and infected ticks was observed in the controls ($P<0.001$). A tick (R. sanguineus) removed from the site of bite on the shoulder of an agricultural worker contained SFG rickettsiae demonstrated by microscopic observation and immunofluorescent staining of hemolymph; seroconversion occurred in the inoculated guinea pig. The dog owned by this patient was also seropositive. All guinea pigs inoculated with hemolymph-positive ticks showed seroconversion after 28 days.

The essential role of the tick is emphasized by the high rate of tick infection (19.7%) and the uneven distribution of SFG rickettsial infection of ticks ranging from 4% to 35% in different areas. These variations correlated with geographic factors: high infection rates in rural areas and lower altitutdes and, conversely, lower infection rates in urban and mountainous areas.

These observations of high proportions of rickettsia-infected ticks and seropositive dogs in areas of Sicily with higher incidence of BF are similar to the evidence for greater rickettsial activity in dogs in France and the USA in areas of higher human attack rates for BF and Rocky Mountain spotted

fever, respectively.

There was an even higher rate of SFG rickettsial infection of ticks from dogs that were associated with human cases of BF in Sicily than the average rate of tick infection; 73% of such dogs had SFG rickettsia-containing ticks. The stimulation of seroconversion of guinea pigs inoculated with tick-rickettsia suspensions strongly suggests that these SFG rickettsiae were, in fact, pathogenic R. conorii. This association of R. conorii-infected R. sanguineus with human BF in western Sicily was most conclusively documented by the isolation of a SFG rickettsia from a R. sanguineus tick which was removed from a patient with BF.

A manuscript reporting four cases of boutonneuse fever that occurred during the cold season of the year has been published in collaboration with investigators at the University of Palermo and Dr. Raoult of Marseille. These documented cases occurred in Sicily and France between November and February. It cannot, therefore, be assumed that boutonneuse fever is limited to the warm weather seasons of the year. The diagnosis must be considered for febrile patients in the Mediterranean basin throughout the year.

In vitro experiments on the pathogenic mechanism or mechanisms of R. conorii have employed the parabiotic chamber model and the plaque model. Parabiotic chambers were employed to determine whether any soluble rickettsial product would injure uninfected cells sharing the same culture medium with cells infected and killed by R. conorii.

The hypothesis that cell and tissue injury are mediated by a rickettsial toxin has been suggested although an exotoxin has never been demonstrated and rickettsial lipopolysaccharides do not have potent toxic activity. Much of the confusion concerning rickettsial pathogenesis is the result of the name given to the phenomenon of the lethal effect of large doses of viable rickettsiae when inoculated intravenously into mice. Traditionally, this rickettsial laboratory assay has been termed the "mouse toxin phenomenon" although it cannot be produced by rickettsiae that are metabolically inactive or dead, and this toxicity has never been produced by a purified component of rickettsiae.

Pairs of sterile parabiotic chambers (Bellco Galss, Vineland, NJ) were separated by 25 mm diameter cellulose triacetate membrane filters (Gelman Sciences, Ann Arbor, MI) with 0.2 um pore size sealed between the chambers with silicone stopcock grease. Coverslips measuring 10.5 x 35 mm were placed in each chamber and were seeded with 5×10^5 VERO cells (CDC Tissue Culture Unit, Atlanta, GA). After incubation at 37°C in minimum esential medium with 5% heat-inactivated fetal calf serum and 10% tryptose phosphate broth for 24-48 hours, monolayers were confluent. The medium was removed, and 36-360 plaque forming units of R. conorii (Malish 7 strain) were inoculated into one chamber of parabiotic chambers. After 30-45 minutes for adsorption of inoculum, 10 ml of the same medium was added. Control pairs of chambers were not inoculated with rickettsiae. Coverslips from adjoining inoculated and uninoculated chambers were examined for evidence of cell death as determined by trypan blue staining and for presence and distribution of R. conorii by direct immunofluorescence. Uninoculated pairs of chambers were examined as controls on day 7 after inoculation. For a positive toxin control, one chamber of each of two pairs was inoculated with a fresh clinical isolate of Pseudomonas aeruginosa with examination of chambers on day 3 and on day 5 by trypan blue staining. The coverslips were examined on days 5 and 6 by phase contrast microscopy after trypan blue staining and then after acetone fixation by direct immunofluorescence for rickettsiae.

The monolayers infected with R. conorii showed severe cytopathic effect

but with less necrosis than R. rickettsii-infected monolayers although nearly all of the cells were infected. In contrast, the adjacent uninoculated monolayers appeared without cytopathic effect. Validation of the parabiotic chamber toxin model was provided by demonstration of progressive destruction of the monolayers in the chamber infected with P. aeruginosa and in the uninfected chamber when examined on days 3 and 5.

The plaque model has been useful in the investigation of the role of the phospholipase-associated rickettsial entry mechanism in cell injury by R. conorii. We have used VERO cell monolayers and R. conorii (Malish 7 strain). Two different drugs that have been reported to possess phospholipase A_2 inhibitory capability, phentermine and indomethacin, were incorporated into the overlay media. Phentermine was added to both the first and second overlay media in the following final concentrations: 0.1 mg/ml, 0.2 mg/ml. 0.4 mg/ml, 0.8 mg/ml, and 1.6 mg/ml. Plaques were counted on day 6 after inoculation. The results are shown in Table 5. Indomethacin was incorporated in both overlay media in final concentraions of 10^{-3}M, 10^{-4}, 10^{-5}M, and 10^{-6}M. Plaques were counted on days 6 and 7. The results are presented in Table 6. In an attempt to determine whether the effect of one of these drugs with phospholipase A_2 inhibitory activity, phentermine, acts on a rickettsial activity or on a host cell activity, the compound was incubated with either R. conorii or VERO cell monolayers prior to inoculation. The rickettsiae were then diluted 10^{-2} such that the final concentration of phentermine at the time of inoculation was negligible. The pretreated monolayer was washed with 3 ml of sterile phosphate buffered saline prior to inoculation to remove unbound phentermine. Plaques were counted on day 7 after inoculation of R. conorii. The results are shown in Tables 7 and 8. Uninfected monolayers showed no toxicity of phentermine pretreatment over the range of 0.1 - 4.0 mg/ml concentration. There was partial toxicity at 10 mg/ml and complete destruction of the monolayer at 20 mg/ml and above.

Because recent studies in our laboratory have shown that synthetic diamidine type protease inhibitors have the ability to block plaque formation by R. rickettsii, we tested the effect of the most effective compound on the R. conorii plaque model. Bis(5-amidino-2-benzimidazolyl)methane, (BABIM), was first incorporated into both first and second overlay media at concentrations of 10^{-4}M, 10^{-5}M, and 10^{-6}M. Plaques were counted on day 6 after inoculation. The results demonstrated that this inhibitor of trypsin-like proteases blocked plaque formation at 10^{-4}M and 10^{-5}M concentrations (Table 9).

R. conorii plaques have been fixed for electron microscopy, embedded in Epon-Araldite, removed from the flask while the topography of the plaque was maintained, and reembedded in Epon-Araldite for sectioning perpendicular to the plane of the monolayer.

Transmission electron microscopy of R. conorii plaques was performed on monolayers maintained in the original topographic orientation. The results were substantially similar to those originally reported for R. rickettsii including cytopathic effect consisting of dilated rough endoplasmic reticulum, rickettsiae trapped within islands of cytosol demarcated by the dilated endoplasmic reticulum, the location of rickettsiae in cytosol free from host membrane-bound vacuoles, and release via filopodia. The most striking contribution of the ultrastructure was a large set of beautiful illustrations of rickettsiae in various stages of emerging from the host cell.

Investigations of cell culture models of R. conorii injury included evaluation of the effect of temperature on cell injury by R. conorii.

Plaques were not appreciably different in quantity at 32°, 34° and 36°C. At 38°C fewer R. conorii plaques were observed.

Some effort has been expended on review and analysis of the published medical and scientific literature on rickettsial disease problems. This effort has been useful in focusing on the critical issues that these diseases pose. A major product of this work is the writing of a comprehensive chapter for a CRC Handbook on viral hemorrhagic fevers and rickettsial diseases edited by Dr. J.H.S. Gear. The chapter entitled "Pathology and Pathogenesis of the Hemorrhagic State in Viral Hemorrhagic Fevers and Rickettsial Diseases" encompasses correlation of clinical and hemorrhagic manifestations, human and experimental animal pathologic lesions, and investigations of hemostatic and pathogenic mechanisms in these diseases. A second major work on synthesis of the current state of knowledge is the planning and editing of a CRC Handbook, Biology of Rickettsial Diseases. The book is a comprehensive work to encompass the meaning of the recent advances in rickettsiology and their implications for understanding rickettsial ecology, physiology, immunity, pathology, and clinical diseases. The field lacks a book that presents its interesting problems as they currently exist in a form that transcends the individual article or collection of research publications. The results should be useful for military medical planning and includes contributions from rickettsiologists with a perspective of rickettsiae that includes military significance.

Conclusions

This research project has succeeded in answering most of the questions posed originally in the contract proposal. The following are conclusions related to the technical objectives:

1. The mechanism of tissue injury in the tache noire of boutonneuse fever is vascular damage with a principal consequence being local accumulation of edema.

2. The ultrastructural cytopathology in the taches noires is cellular necrosis.

3. The human target cells of R. conorii in the tache noire are endothelial cells primarily with some macrophages apparently also containing rickettsiae. These results were obtained by direct immunofluorescence since too few rickettsaie were detected ultrastructurally for analysis.

4. The cellular location of rickettsiae was judged best in the plaque model, where R. conorii is located free in the cytosol.

5. No quantitative determination of association of R. conorii with ultrastructural cytopathologic and tissue lesions was accomplished owing to the paucity of rickettsiae at the time of biopsy.

6. The host immune and inflammatory cellular response to R. conorii infection in the tache noire is lymphocytes, macrophages, and polymorphonuclear leukocytes, in decreasing order of frequency. There were more T-lymphocytes than macrophages and very few B-lymphocytes. Among the T-lymphocytes, T-helper/inducer subset was 1.4 times more numerous than T-

cytotoxic/suppressor subset.

7. The pathogenic mechanism of R. conorii in the plaque model was direct rickettsial cytopathic effect and was blocked by compounds that have been reported as phospholipase and protease inhibitors.

The results of the specific hypotheses originally proposed and tested are the following:

1. Tissue injury in the tache noire is not caused by thrombosis.

2. Injured target cells in the plaque model did contain dilated rough endoplasmic reticulum.

3. The target cells of R. conorii in the tache noire appear to be endothelium >> phagocytes.

4. Too few rickettsiae were identified in taches noires to evaluate the cellular location of rickettsiae critically.

5. The same problem prevented analysis of association of intracellular rickettsiae with cytopathologic ultrastructure.

6. Both thrombi and rickettsiae were too few for ultrastructural analysis. Thrombi are, in fact, not pathogenetically important.

7. Foci of injury contained more T-lymphocytes than macrophages. T-helper/inducers outnumbered T-cytotoxic/suppressors. PMN's and B-lymphocytes were few.

8. R. conorii is less cytopathic than R. rickettsii, but the cytopathic effect, when present, is similar.

9. R. conorii plaques are inhibited by the phospholipase inhibitors phentermine and indomethacin.

10. R. conorii plaqued more efficiently at 32-36°C than at 38°C.

11. No exotoxin of R. conorii was detected in the parabiotic chamber model.

The currently recognized high rate of R. conorii infections in Spain, Portugal, France, Israel, and Italy is likely to continue. The absence of data from northern, eastern, and western Africa and the Middle East reflect the failure of clinical and epidemiologic systems in these areas to focus the necessary laboratory methods and organizational efforts on the problem. The disease is likely to be or to become an important unrecognized cause of incapacitating febrile illness in these areas. Boutonneuse fever is a neglected disease that has recently attracted investigation in Sicily, Marseille, and Salamanca. Collaborative relationships with these laboratories encourages study of the epidemiologic, clinical, pathophysiologic, and rickettsiologic questions at a admirable level of effort and expertise. This contract has fostered a significant part of this result and can continue to play an important role in maintaining enthusiasm for elucidating the unknown factors that boutonneuse fever encompasses.

It is probable that R. conorii infections are a greater cause of morbidity and mortality than is now appreciated. In Marseille, Salamanca, Israel, and South Africa, severe illness is regularly recognized in a substantial portion of those affected. The application of immunofluorescence to autopsy specimens is revealing the rickettsial and clinicopathologic basis for the pathophysiologic observations such as meningoencephalitis, hepatitis, and other visceral involvement. The documentation of hepatic lesions in seven consecutive Sicilian patients with hepatic biopsies during boutonneuse fever demonstrates the serious nature of the disease. The recognition that boutonneuse fever may cause an incapacitating febrile illness with neither rash nor tache noire suggests that the high incidence of antibodies to R. conorii in various populations may very well be due to prior undiagnosed symptomatic R. conorii infection. Specific diagnostic tests are not applied to patients who do not present at least some of the classic manifestations of the disease, particularly a rash. Such a situation is obviously a potential threat to military health of soldiers in the wide geographic distribution of R. conorii. This situation is analogous to the problem of scrub typhus in World War II, e.g. the Assam-Burma theater of operations.

Particular problems that have been identified recently are the frequent hepatic involvement, lifethreatening gastrointestinal hemorrhage, severity of illness in glucose-6-phosphate dehydrogenase deficient men even if they are young and previously healthy, the occurrence of cases even in cold weather months, and occurrence of familial cases that suggest "hot spots" of R. conorii endemicity.

Our careful study of the tache noires has elucidated important factors in the pathogenesis that conform to current clinical features of R. conorii pathogenesis: 1) R. conorii is present in the lesions and appears to play a direct cytopathic role in vascular injury, 2), vasculopathic edema is a major pathophysiologic effect, 3) ischemic necrosis is not necessarily the end result of vascular injury, 4) thrombosis is not the major pathologic effect of vascular injury and, thus, should not be treated with anticoagulation, and 5) in human disease the local antirickettsial immune and inflammatory response is predominantly lymphocytes and macrophages.

Electron microscopy and immunofluorescence have not demonstrated large quantities of R. conorii in tache noires or liver biopsies. The possible explanations include: 1) the host immune and phagocytic response in these lesions may be effective at the stage of illness when patients are referred for tertiary subspecialty medical care, 2) antirickettsial antimicrobial therapy may have been administered prior to collection of specimens, 3) ultrastructural sampling may be a problem.

Recommendations:

1. Offer skin biopsy immunofluorescent demonstration of R. conorii in tache noire as a reference military laboratory diagnostic test for boutonneuse fever.

2. Study the rickettsial hepatitis of boutonneuse fever and experimental R. conorii infection in animal models of visceral tissue injury by R. conorii.

3. Initiate a search for nonpathogenic R. conorii or R. conorii-like isolates in ticks in Sicily.

4. Develop a more rapid, quantifiable assay system for growth of and cell injury by R. conorii for screening amidine type trypsin-like protease inhibitors as potential prophylactic or adjunctive therapeutic agents.

5. Study amidine type protease inhibitors for inhibition of cell injury by R. conorii.

6. Continue in vitro study of pathogenic mechanisms of cell injury by R. conorii.

7. Initiate epidemiologic surveillance of troops in Spain, Italy, and other places in the geographic distribution of R. conorii for acute cases of boutonneuse fever and for seroprevalence of antibodies to R. conorii.

8. Obtain seroepidemiologic data on the prevalence of R. conorii infection in indigenous populations in Africa and the Middle East.

9. Study the capability of our monoclonal antibodies to block cell injury or infection by R. conorii.

10. Study the clinical and in vitro mechanisms of enhanced severity of boutonneuse fever in glucose-6-phosphate dehydrogenase deficient subjects and the role of hemolysis in this problem.

11. Exploit the antigen-producing genomic library of R. conorii recombinant DNA to determine rickettsial pathogenic mechanisms.

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Table 1

Evaluation of Taches Noires from Sicilian Patients with Confirmed Rickettsia conorii Infection

Patient	Cutaneous Necrosis	Vasculitis	Thrombosis	Dermal Edema	Predominant[a] WBC	IF[b] R. conorii
1	+++[c]	+++	+	++	L,M	+
2	0	+++	0	++	L,E	+
3	+	+++	0	++	L,M	+
4	++	+++	++	++	L,M	+
5	0	+++	+	++	L,M	+
6	0	++	0	++	L	0
7	0	++	+	+	L	+
8	+++	++	+	++	L	+
9	0	++	0	++	L	0
10	+++	++	0	++	L	0
11	+++	+++	0	++	M	0
13	+++	+++	+++	++	L,P	+
14	+++	+++	0	+	L	+
16	+++	+++	0	+	L	+
17	NE[d]	++	0	++	L	+
18	+++	+++	0	+	M,L	+

a - Predominant perivascular leukocytes: L, small lymphocyte; M, large mononuclear cell: E, eosinophil; P, PMN
b - Presence (+) or absence (-) of immunofluorescent Rickettsia conorii
c - Absent (0): +, mild; ++, moderate; +++, severe
d - Feature not available for evaluation

TABLE 2: EVALUATION OF THE CELLULAR CONTENT OF THE PERIVASCULAR INFLAMMATORY INFILTRATE IN TACHES NOIRES

Patient	Day[a]	IFA titer at bx	IFA titer convalescent	DFA R. conorii	% Cells[b] T-cells	% Cells[b] B-cells	% Cells[b] Macrophages	T-helper-inducer/T-cytotoxic-suppressor ratio
1	7	1:20	1:1280	0	62.96	10.39	26.63	1.659
2	6	1:20	1:1280	+	63.20	20.06	16.72	1.362
3	7	<1:20	1:2560	+	52.06	19.04	28.89	1.346
4	11	1:20	1:2560	+	59.39	12.18	28.42	1.449
5	3	1:20	1:640	+	44.80	21.89	33.29	1.285
6	2	<1:20	1:640	+	51.85	22.21	25.92	1.298

a - Day when the biopsy was taken (from the onset of fever)
b - Percentage of the total number of cells counted

Table 3
Clinical and Serologic Data for Sicilian Patients with Boutonneuse Fever Undergoing Liver Biopsy

Patient	Age/Sex	Tache noire	Rash	Fever	Serology Acute	Serology Convalescent
1	43M	+[a]	0[b]	+	160[c]	N.D.[d]
2	74M	+	+	+	160	160
3	66F	+	+	+	40	N.D.
4	67F	+	+	+	N.D.	320
5	85F	+	+	+	40	160
6	68M	+	0	+	40	N.D.
7	50F	+	+	+	320	1280

a - present
b - absent
c - reciprocal of indirect immunofluorescent antibody titer against R. conorii
d - not determined

Table 4
Evaluation of Sicilian Patients with Boutonneuse Fever for Hepatic Involvement

Patient	LDH[a]	ALT[b]	AST[c]	AP[d]	GGT[e]	Bil[f]	day of bx[g]	Lesions	IF R. conorii
1	120	15	22	139	15	normal	1	+[h]	0[i]
2	259	16	20	N.D.	51	normal	3	+	0
3	390	38	30	152	normal	1.1	6	+	0
4	251	10	14	N.D.	N.D.	0.9	13	+	0
5	255	28	18	290	18	normal	2	+	0
6	210	21	18	199	N.D.	normal	2	+	0
7	N.D.	85	93	477	160	1.0	30	+	0

a - serum lactate dehydrogenase (reference interval, 100-240 IU/l)
b - serum alanine aminotransferase (reference interval, 6-31 IU/l)
c - serum aspartate aminotransferase (reference interval, 6-31 IU/l)
d - serum alkaline phosphatase (reference interval, 60-170 UI/l)
e - serum gamma glutamyl transpeptidase (reference interval, 4-18 IU/l)
f - total serum bilirubin (reference interval, less than 1.0 mg/dl)
g - number of days after onset of fever
h - presence of hepatic lesions
i - absence of immunofluorescent R. conorii

Table 5

Effect of Phentermine on Plaque Formation by *Rickettsia conorii*

Inoculum	Concentration of Phentermine (mg/ml)					
	None	0.1	0.2	0.4	0.8	1.6
R. conorii	42.5±5.2[a]	40.0±4.8	30.3±6.0	16.3±2.5	14.0±2.2	Toxic
None	0	-	-	-	-	Toxic

a - Number of plaques/flask (mean ± standard deviation)

Table 6

Effect of Indomethacin on Plaque Formation by *Rickettsia conorii*

Conc. Indomethacin	*R. conorii*		None	
	Day 6	Day 7	Day 6	Day 7
None	13.5±0.0[a]	15.3±8.0	0	0
10^{-6}M	12.3±9.6[b]	13.5±3.1	-[c]	-
10^{-5}M	0±0	0±0	-	-
10^{-4}M	0±0[d]	0±0[d]	-	-
10^{-3}	Toxic[e]	Toxic	Toxic	Toxic

a - Number of plaques/flask (mean ± standard deviation)
b - Plaques smaller than untreated *R. conorii* plaques
c - Not done
d - Partial destruction of monolayer by toxicity of indomethacin; no plaques in the intact portions of monolayer
e - Indomethacin toxic to monolayer at this concentration

Table 7

Effect of Phentermine Pretreatment of *Rickettsia conorii* on Plaque Formation

Inoculum (Phentermine Concentration)		Plaques[a]
R. conorii	(none)	TNTC
R. conorii + phentermine	(20 mg/ml)	TNTC
R. conorii + phentermine	(40 mg/ml)	TNTC
R. conorii + phentermine	(80 mg/ml)	15.8±4.3
R. conorii + phentermine	(320 mg/ml)	0±0

a - Number of plaques/flask ± standard deviation

Table 8

Effect of Phentermine Pretreatment of Vero Cells on Subsequent Plaque Formation by *Rickettsia conorii*

Conc. of Phentermine in Pretreatment	Plaques[a]
None	20.8±11.5
1 mg/ml	38.8±2.5
4 mg/ml	64.0±14.1
10 mg/ml	[b]

a - Number of plaques/flask ± standard deviation
b - Partial destruction of monolayer by toxicity of phentermine; estimated adjusted mean of plaque counts, 86.5

Table 9

Effect of BABIM on Plaque Formation by *Rickettsia conorii*

Inoculum	Treatment (conc.)	Plaques
R. conorii	None	21.8±18.1
R. conorii	BABIM (10^{-4}M)	0±0
R. conorii	BABIM (10^{-5}M)	0±0
R. conorii	BABIM (10^{-6}M)	21.8±19.1
None	None	0±0
None	BABIM (10^{-4}M)	0±0
None	BABIM (10^{-5}M)	0±0
None	BABIM (10^{-6}M)	0±0